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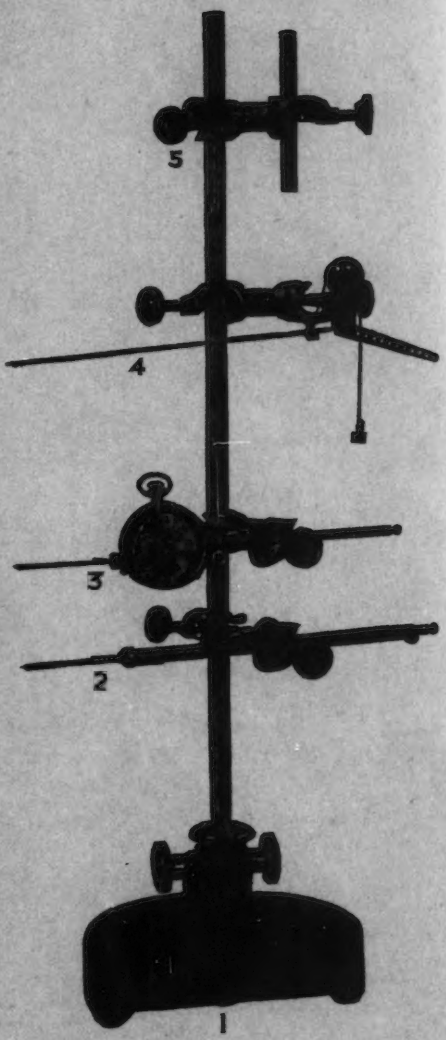

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THE EFFECT OF MIXTURES OF TISSUE EXTRACTS AND BLOOD SERA OF VARIOUS ANIMALS ON THE COAGULATION OF BLOOD

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Loeb (1, 2) Hewlett (3), Muraschew (4), and Nolf (5) have shown that the tissue coagulins, (thrombokinase of Morawitz, tissue fibrinogen of Wooldridge and Mills, thromboplastic substances of Alex Schmidt and Howell, cytozyme of Fuld, Spiro and Bordet) are specific in that the plasma of one group of animals is more efficiently coagulated when tissue coagulin from the same group is used (mammals, birds, reptiles, amphibia, invertebrates). This relationship between a certain plasma and tissue coagulin Loeb designated as a specific adaptation to distinguish it from other kinds of specificity. With erythrocytes (6) this specific adaptation was more pronounced than with other cells or tissues. Here a specific adaptation could be demonstrated even between different mammalian species.

It had been previously noted, especially by Morawitz, that tissue extracts and blood sera when mixed, were more effective in causing coagulation of blood than either of these substances alone. In addition to this accelerating effect, Loeb (7), and subsequently Loeb, Fleisher and Tuttle (8), found that mixtures of blood sera and tissue extracts produced inhibition of coagulation under certain conditions. The amount of inhibition varied with different sera. It was, for instance, very pronounced if dog serum was used; less in the case of other sera. Furthermore, the inhibition was roughly proportional to the time of incubation of serum and extract. Long periods of incubation entirely prevented coagulation in some in-

stances. Short periods of incubation, on the other hand, frequently resulted in an acceleration of coagulation. The inhibiting effect was much more manifest with hirudin or peptone plasma, while the accelerating effect preponderated with fluoride plasma. Erythrocyte stroma, acting on blood plasma alone, caused acceleration similar to that noted with other organ extracts. However when erythrocytes were incubated with blood serum, the inhibiting effect was almost entirely lacking.

Evidence indicated that the interaction between serum and tissue extract, with the formation of both inhibiting and accelerating substances, depended on specifically adapted substances in the serum and extract. Superimposed upon the specific character of these substances in the sera and extracts, were differences in absolute quantities present in various sera. Thus dog serum seemed to contain the largest quantity of the inhibiting substance. In addition to the evidence of specificity in the interaction of inhibiting substances, Loeb (7), and Loeb, Fleisher and Tuttle (8), found a specific relation between the tissue extract (tissue coagulin) and the coagulable substance in the plasma.

The direct action of tissue coagulin on invertebrate blood can be proven, and the essential factors for the coagulation of invertebrate and vertebrate bloods are very similar. From this and other facts observed in the coagulation of vetebrate blood, Loeb assumed that acceleration of coagulation may occur either from interaction of tissue extract and blood serum, or from the separate direct action of tissue coagulin and thrombin (blood coagulin) on fibrinogen.

Mills and Stewart Mathews (9) in 1922 showed that a mixture of tissue extract and rabbit serum was at first much more active in coagulating citrated horse plasma than was serum alone. After standing, this mixture lost most of its coagulating power. In Loeb's experiments it should be noted that fluoride plasma favored accelerating rather than inhibiting effects in mixtures of serum and extract, and it is known that in many respects citrate plasma behaves like fluoride plasma.

The interaction of blood sera and tissue extracts which leads to an inhibition of blood coagulation became of interest in another direction. Dold (10) observed that intravenous injection of tissue extract was poisonous for animals, and that previous incubation of the extract with blood serum diminished or abolished this toxic action. Loeb (11) interpreted this phenomenon as due to the inhibition of blood coagulation which follows the mixing and incubation of serum and tissue extract before injection. In accordance with this interpretation, Dieckmann (12) found that the detoxicating effect which the serum of pregnant women had on placental extracts, and which was lacking when the serum of eclamptic women was used, was closely paralleled by the in vitro effect of these mixtures on coagulation. In vitro also, mixtures of placental extracts and normal

blood serum (of non-pregnant women) produced inhibition of coagulation, while with eclamptic serum no such inhibition occurred.

As to the origin of the inhibiting substance present in normal blood serum, Loeb (7) found that it was lacking, or at least diminished, in dogs whose livers showed marked degenerative changes as a result of phosphorus poisoning.

The complexity of conditions and multiplicity of factors in the interaction of blood serum and tissue extracts made it desirable to extend the previous investigations to a greater variety of extracts and sera. Data concerning the quantitative differences and the specific adaptation of inhibiting and accelerating substances in these sera and extracts have been collected. We have avoided the use of blood plasma prevented from spontaneous clotting by inactivation of calcium. Instead we used Howell's (13) heparin to prevent coagulation. Heparin plasma corresponds closely to the hirudin plasma which Loeb and his associates used.

METHOD. The amount of serum used in every case was 0.7 cc., the amount of extract 0.3 cc., and the amount of plasma 1 cc. The periods of incubation in all experiments were 0, 1, 3, 5, 10, 20, 40, and 80 minutes. Tissue extract was prepared by extracting dried pulverized kidneys with 0.9 per cent NaCl solution. The concentration of extract was adjusted so that coagulation of heparinized dog plasma would occur in 3 to 5 minutes.

In each experiment blood sera and kidney extracts were first mixed and then allowed to incubate for the periods indicated above. At the end of each period blood plasma was added and the time required for coagulation observed. In control experiments 0.7 cc. of 0.9 per cent NaCl solution was substituted for blood serum. Dog plasma was used in the first group of experiments. It was prepared by adding heparin in the proportion of 1 mgm. per 5 cc. of blood.

For the sake of brevity the following abbreviations are used in this paper:

Dog and goose plasma, DP and GP respectively.

Dog, human, rabbit, ox, sheep, and goose sera, DS, HS, RS, OS, SS, and GS respectively.

Dog, human, rabbit, ox, sheep, and goose kidney extracts, DKE, HKE, RKE, OKE, SKE, and GKE respectively.

Effect of combinations of dog serum (DS) and various extracts on coagulation of dog plasma (DP). Five different kidney extracts, DKE, HKE, SKE, OKE, and RKE were used in combination with DS.

1. *DKE plus DS.* Without exception marked inhibition of coagulation occurred when DKE and DS were allowed to incubate together. In some experiments an initial acceleration was observed after 1 to 3 minutes standing, while in other cases inhibition was present from the beginning. Chart 1.

2. *OKE plus DS.* Usually a rather marked inhibiting effect was manifest with this combination. An initial slight acceleration, especially after 1 minute incubation, was noted regularly. Chart 1.

3. *SKE plus DS.* A moderate inhibition of coagulation was noted in all experiments in which SKE and DS were incubated together. This was preceded in some cases by an initial acceleration, present after 1 minute and absent after 3 minutes' incubation. Chart 1.

4. *HKE plus DS.* Moderate inhibition, without initial acceleration, was noted with this combination. Chart 1.

5. *RKE plus DS.* Combinations of RKE and DS inhibited coagulation to about the same degree as SKE and HKE with DS. An initial acceleration was noted in some cases, but generally inhibition was well marked at an early stage. Chart 1.

Effect of combinations of human serum (HS) and various extracts on the coagulation of dog plasma (DP). Two kidney extracts, DKE and HKE were combined with HS and their effects on the coagulation of DP noted.

1. *DKE plus HS.* In most experiments only a relatively slight inhibition of coagulation occurred when DKE and HS were incubated together. The average of all experiments is indicated on chart 2. Because of the marked inhibition occurring in some experiments, this line indicates a greater value than that actually found in the majority of cases.

2. *HKE plus HS.* Varying degrees of inhibition were noted when HKE and HS were incubated together. After 80 minutes incubation, the greatest inhibition was 76 minutes; the least 8 minutes; while in most experiments it was 20 to 25 minutes. Initial acceleration was observed in only a few cases. Chart 2.

Effect of combinations of sheep serum (SS) and various extracts on the coagulation of dog plasma (DP). Three kidney extracts, HKE, DKE, and SKE were used in combination with SS.

1. *HKE plus SS.* A moderate inhibition of coagulation developed when HKE and SS were incubated together. In some cases an initial acceleration occurred after 1 minute of incubation. Chart 3.

2. *DKE plus SS.* Less inhibition of coagulation was noted with DKE plus SS than with HKE plus SS, although there was always a definite delay of coagulation after longer periods of incubation. Chart 3.

3. *SKE plus SS.* In most cases a very marked acceleration of coagulation occurred with SS and SKE, persisting even after 80 minutes' incubation. In other cases initial accelerative changes were followed by mild inhibitory effects. This latter change was noted expecially when weaker extracts were used. Chart 3.

Effect of combinations of ox serum (OS) and various extracts on the coagulation of dog plasma (DP). Three kidney extracts, HKE, DKE and OKE, were used in combination with OS.

1. *HKE plus OS.* A slight but definite inhibition of coagulation was noted when HKE and OS were incubated together. This mixture produced inhibition even without incubation. Chart 4.

2. *DKE plus OS.* A similar slight inhibition was manifest when DKE and OS were incubated together. An initial acceleration was usual after 1 and 5 minutes' incubation. Chart 4.

3. *OKE plus OS.* In most cases a distinct acceleration of coagulation occurred with this combination even after 80 minutes' incubation. In a few isolated cases mild inhibition was noted after the longer periods of incubation. Chart 4.

Effect of combinations of rabbit serum (RS) and various extracts on the coagulation of dog plasma (DP). Three kidney extracts, HKE, DKE, and RKE, were used in combination with RS.

1. *HKE plus RS.* Incubation of HKE and RS caused moderate inhibition of coagulation. This was preceded in all cases by an initial acceleration. Chart 5.

2. *DKE plus RS.* Variable effects on coagulation resulted from incubation of DKE and RS. In some experiments marked acceleration occurred, others showed slight inhibition, while another group showed marked inhibition. Chart 5.

3. *RKE plus RS.* In this combination too, irregular results were obtained, sometimes acceleration and sometimes inhibition of coagulation occurring. Chart 5.

In the foregoing group of experiments, only extracts and sera of mammalian species were used. Extracts and sera from birds were now employed to determine the relationship between animals phylogenetically more widely separated. Thus combinations of GS were made with GKE and DKE, and of DS with GKE and DKE, using both goose and dog plasma. In these experiments the use of tissue extracts on heterologous plasma substantiated Loeb's former observation that more efficient coagulation occurs when extracts are allowed to act on plasma of the same species.

Effect of goose serum (GS) on goose and dog kidney extracts (GKE and DKE), using goose plasma (GP). 1. *GS plus DKE.* DKE was very inefficient in causing coagulation of GP. Thus when combinations of GS and DKE were allowed to incubate, clotting occurred very slowly, but at a rate only slightly slower than the control. This indicates of course, that GS has little inhibiting effect on DKE.

2. *GS plus GKE.* Combinations of GS and GKE showed most striking inhibitory effects which occurred even after 1 minute of incubation and increased gradually with prolonged standing.

Effect of dog serum (DS) on dog and goose kidney extracts (DKE and GKE), using goose plasma (GP). 1. *DS plus DKE.* The action of these mixtures following incubation was somewhat difficult to interpret owing to the

slow action of DKE on GP. However a slight inhibition of coagulation was always noted after longer periods of incubation.

2. *DS plus GKE.* Combinations of DS and GKE showed very little inhibitory effect even after long periods of incubation.

Effect of goose serum (GS) on goose and dog kidney extracts (GKE and DKE), using dog plasma (DP). 1. *GS plus GKE.* Using DP instead of GP, marked inhibition was again noted when GS and GKE were incubated together. Chart 6.

2. *GS plus DKE.* GS in combination with DKE influenced the clotting time of the latter very little. Chart 6.

Effect of dog serum (DS) on dog and goose kidney extracts (DKE and GKE) using dog plasma (DP). 1. *DS plus DKE.* Combinations of DS and DKE gave the usual marked inhibitory effects observed so frequently in the preceding experiments.

2. *DS plus GKE.* Combinations of DS and GKE were very inactive in altering the coagulation time.

The effect of phosphorus poisoning on the inhibiting properties of dog serum. The origin of the inhibiting precursor substances found in the blood serum of various species was of interest. Previous work (2) had pointed to the liver as an organ of particular importance in blood coagulation. Howell and Holt (13) prepared heparin from the liver, a substance which inhibits coagulation of blood apparently in a complex manner. Loeb (7) showed that the precursor inhibiting substance is much diminished or altogether lacking in the serum of dogs poisoned by oral administration of phosphorus. We modified these earlier investigations, injecting the phosphorus subcutaneously.

Yellow phosphorus was dissolved in carbon disulphide in an atmosphere of CO_2. To this solution sufficient mineral oil was added to make a 0.4 per cent suspension. The CS_2 was driven off by bubbling CO_2 through the mixture. Two cubic centimeters of this suspension per kilo body weight were injected subcutaneously into the loose abdominal tissue of six dogs. Five of these dogs died within 24 hours after the injection. Autopsy showed extensive necrosis and cloudy swelling of the liver and kidneys, large hemorrhages into the lungs, and occasionally hemorrhages into the bowel.

The sera of the five dogs which died showed complete loss of inhibiting power when mixed with tissue extracts (table 1). The serum of one dog, which did not die as early and which developed an ulcer at the site of injection, showed some inhibition similar to normal serum.

In one experiment the incubation period and the amount of tissue extract were kept constant, but the amount of serum varied. In this case, increase in the amount of normal serum produced a proportional increase in inhibition of coagulation, whereas no inhibiting effect was noted with serum of the phosphorus poisoned dog.

We may then conclude that phosphorus poisoning produced changes in the bodies of dogs which destroyed or inactivated inhibiting precursor substances. Inasmuch as the liver was most severely affected, this organ may be concerned in the loss of inhibiting power.

DISCUSSION AND CONCLUSIONS. These experiments are in agreement with the conclusions of Loeb that when tissue extracts and blood sera are incubated together, two factors determine the effect of these mixtures on coagulation of blood plasma. One of these factors accelerates coagulation beyond that which occurs with tissue extracts alone, and the other inhibits coagulation. Accelerating and inhibiting factors are both present in a given mixture of serum and extract, and the time required for coagulation may be taken as a resultant of these two forces.

TABLE 1

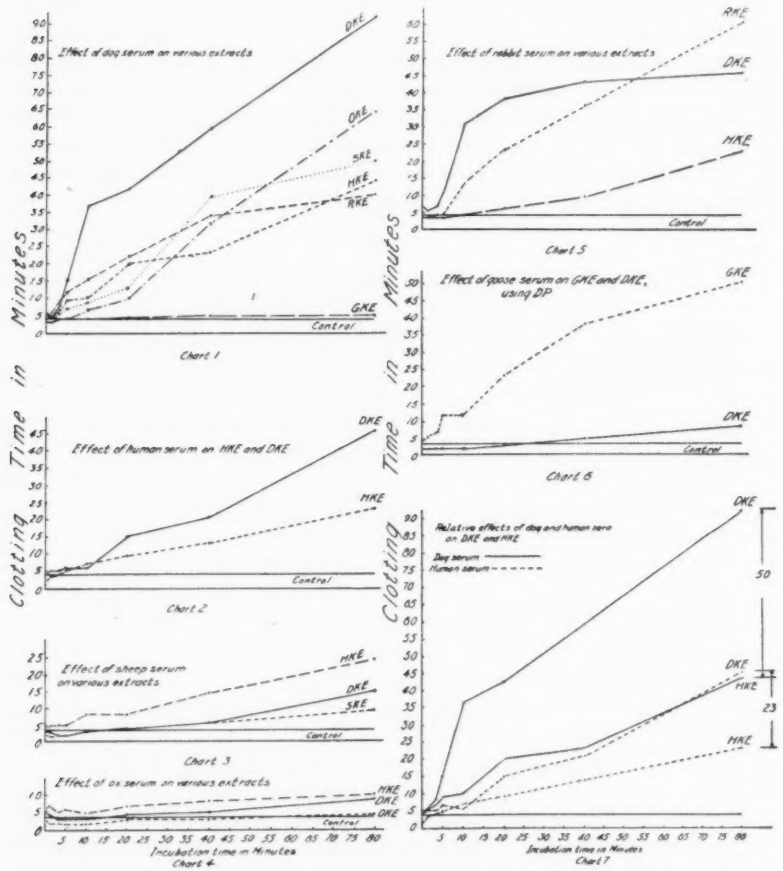
Comparative effects of DKE + serum of normal dogs + DP, and DKE + serum of dogs previously injected with phosphorus + DP

1 cc. DP + 0.7 cc. normal and phosphorus serum + 0.3 cc. DKE

	INCUBATION TIME					CONTROL
	0	5	10	20	25	
Normal serum	1.5	1.5	30	60	60	2
Phosphorus serum, dog 1	1.5	2	2	3	3.5	2
Normal serum	3.5	4	12	17	25	2
Phosphorus serum, dog 2	2	2	2	2	2	2
Normal serum	1.5	1.5	29	46	60	2
Phosphorus serum, dog 3	1.5	2	2	2	3.5	2
Normal serum	3	5	26	60	60	3
Phosphorus serum, dog 4	3	2	2	5	7	3
Normal serum	4	6	15	21	37	3
Phosphorus serum, dog 5	3	6	6	7	8	3

Two observations in these experiments tend to substantiate the presence of accelerating factors. First, in many cases there is an initial phase of acceleration after short periods of incubation. Second, mixtures of sheep kidney extract with sheep serum, and ox kidney extract with its homologous serum lead to acceleration of coagulation even after long periods of incubation. However if sheep and ox serum are combined with dog and human extracts, inhibition instead of acceleration occurs. This inhibition, however, is much less pronounced than when these extracts, DKE and HKE, are combined with dog serum. Such a circumstance suggests that the inhibiting and accelerating substances vary in absolute amount or

activity in different mixtures of serum and extract. Thus sheep and ox sera, when combined with various extracts, develop more active or greater amount of accelerating substances than other sera in combination with these same extracts.



Charts 1–7. Graphic illustrations of the effects of sera and tissue extracts on blood coagulation. Lines represent the average of results.

The inhibitory factors are manifest in mixtures of dog and human sera with tissue extracts. Most marked inhibition of coagulation occurs with dog serum, indicating that the absolute amount of inhibiting substance may also vary in different combinations.

Varied effects on coagulation are noted when rabbit serum is combined

with various extracts. Some cases show marked inhibition, others marked acceleration, and still others show intermediate effects. Such variations can be explained by supposing that in individual rabbit sera the amounts of inhibiting and accelerating substances vary.

Loeb suggested that these inhibitions and accelerations were most marked when homologous combinations of serum and extract were used. A specific adaptation, in other words, existed between both inhibiting and accelerating substances, or between their precursors, in the serum and extract. Loeb compared animals as far distant as birds and mammals. Our experiments included combinations of sera and extracts from various mammalian species. Indications of specificity were found even here.

Chart 1 shows that more marked inhibition occurs when dog serum (DS) is combined with dog kidney extract (DKE) than when combined with OKE, SKE, RKE, or HKE. This points to a specific interaction between inhibiting substances in DS and DKE. A relative specificity is apparent when human and dog kidney extracts (HKE and DKE) are combined. Thus while DKE plus HS is more effective in producing inhibition than HKE plus HS, the difference in degree of inhibition is much less between these two combinations than between DKE plus DS, and HKE plus DS (chart 7). The lack of absolute specificity in this case we attribute to the large amounts of inhibiting substances present in DS and DKE.

A similar specificity is noted in experiments where accelerating factors predominate. Thus sheep and ox sera in combination with their homologous extracts, SKE and OKE respectively, produce marked acceleration, while combinations with DKE and HKE are slightly inhibiting.

It is interesting to note that although sheep and ox sera in combination with their homologous extracts produce acceleration of coagulation, each is capable of producing inhibition in heterologous combinations. Thus SKE and OKE in combination with dog serum produce inhibiting effects which in the case of OKE are very marked (chart 1). Similarly combinations of SS and OS with HKE and DKE (charts 3 and 4) cause a mild inhibition of coagulation. Inhibiting precursor substances are present, therefore, in both SS and OS and in SKE and OKE. Thus if accelerating effects prevail with homologous combinations, this must be due to a specific adaptation between the accelerating precursor substances in both sera and extracts.

Cross experiments with avian and mammalian bloods present even more conclusive evidence of specificity. A quite marked inhibition of clotting is noted when GKE and GS are incubated together. But when GKE is incubated with DS, or GS with DKE, very little change in clotting time occurs. This of course, indicates a specific adaptation between serum and extract from the same class of animals. Although the rate of clotting is altered by the kind of plasma used, the specific reaction is manifest with both dog and goose plasma.

SUMMARY

1. Incubation of various blood sera and kidney extracts produce the following effects on coagulation of dog heparinized plasma: a. Marked inhibition of clotting preceded in some cases by acceleration. b. Marked acceleration of clotting.

2. Evidence points to the development of two substances in mixtures of serum and extract, one causing inhibition and the other acceleration of coagulation. Both inhibiting and accelerating substances form through combinations of precursor substances in blood sera and tissue extracts.

3. Accelerating and inhibiting precursor substances are both present in all sera and extracts tested, but they vary in amount or activity in different species. The effect of the mixtures on coagulation is the resultant action of these two substances.

4. There is evidence of a specific adaptation between both accelerating and inhibiting substances.

5. The inhibiting precursor substances tend to disappear in the blood serum of phosphorus poisoned dogs.

The authors are indebted to Dr. Leo Loeb for suggesting this problem and for rendering invaluable assistance during the course of the work.

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THE ULTRAVIOLET ABSORPTION SPECTRUM OF HEMOLYZED BLOOD CORPUSCLES IN RELATION TO RICKETS[1]

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For some time there has been a growing dissatisfaction with the available methods for the quantitative estimation of the extent of the development of rickets in animals. This is forcibly brought out in the work of Maughan (1928) and Maughan and Dye (1930) on the cure of rickets by irradiation with ultraviolet light, in the course of which six different tests were applied to each animal and the average used as a measure of the per cent recovery from the illness. As they point out, any one of these tests provides a fair criterion of the condition of the animal and all run to some degree parallel during the progress of the cure; nevertheless all must be considered in estimating the true condition for comparative purposes, as for example in determining the relative efficiency of different wave lengths. A recent discussion in Nature (anonymous, 1930) elaborates on the analytical difficulties confronting the investigator of rickets. Any method, which could yield a rapid and reliably quantitative determination of the status of the disease and which, incidentally, did not necessitate the killing of the test animal, would be highly desirable.

In this connection, the data obtained by Suhrmann and his collaborators (1929a, b) on the ultraviolet absorption spectra of blood and its various constituents seemed to offer a promising lead. They found, using blood from healthy and rachitic rats, that a characteristic difference existed between the absorption spectra of the hemolized solutions of the washed blood corpuscles from the respective bloods. Since extremely rachitic animals were presumably used in this test, it appeared worth while to investigate the question of a possible parallelism between the extent of this reported change in the absorption spectrum and the degree of rickets as determined by the conventional methods. The nature of the change

[1] The investigations on which this article is based were supported by grants from the Heckscher Foundation for the Advancement of Research, established by August Heckscher at Cornell University.

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observed by Suhrmann was one of intensity and therefore amenable, as he has himself pointed out (1929b), to quantitative treatment. This change was attributed to an alteration in the contents of the blood cell, i.e., of the hemoglobin. But the latter is generally regarded as a single chemical individual and it seems rather questionable that any change in its chemical constitution could occur as the result of a pathological condition like rickets.

Since experimental material was available at the Physiology Department in the form of chickens which had been allowed to develop severe rickets and had then been cured to varying degrees by ultraviolet radiation, we proceeded to investigate the above questions with the apparatus for absorption spectra, which we have been using in the study of chemical problems. Later, when our results with chickens proved to be entirely negative, rats, both healthy and rachitic, were also placed at our disposal by Dr. G. H. Maughan of the Physiology Department. We take this opportunity to thank him again for his coöperation in this matter and for his kindness in drawing the blood samples.

The chickens and rats used in this experiment were in each case all from the same breeding and of the same age and had been carefully selected at the start of the feeding experiment for uniformity of size and general appearance of health. The rats were rendered rachitic by being kept on the Steenbock diet no. 2965:

	per cent
Yellow corn	76
Wheat gluten	20
Calcium carbonate	3
Sodium chloride	1

Ca:P = 4.25:1

They were kept in a room screened from direct daylight and illuminated only by ordinary 40 watt incandescent lamps.

It may be stated at once that the results of our experiments are directly opposite to those of Suhrmann. No detectable difference between the absorption spectra of the blood of healthy and rachitic rats has been observed. Subsequent experiments have led, nevertheless, to a reasonable explanation for the difference reported by him, namely, light scattering due to suspended matter and calculations based on simple physical theory corroborate this hypothesis.

EXPERIMENTAL. The absorption spectra were obtained with a Hilger quartz spectrograph (C_1) equipped with a Hilger rotating sector photometer. A description of the local apparatus and technique, as well as further references, is given by Orndorff, Gibbs and McNulty (1925). The light source was an underwater spark between brass electrodes, which

was operated by means of a high frequency, high potential current from a Tesla transformer. This gives a perfectly continuous spectrum through the entire ultraviolet region. Suhrmann, whose experimental procedure for the measurement of absorption consisted in determining, by means of a photoelectric cell, the change in intensity of *weak* monochromatic light on passing through the solution, has made a general criticism of the method of photographic photometry on the ground that the blood solutions change when exposed to strong light. This is undoubtedly true if the exposure is sufficiently long, but no such effect was observed under our experimental conditions. This may be ascribed to the relatively short exposures which suffice to give the necessary degree of blackening on the photographic plate with the above apparatus. For the series of 13 to 15 exposures, corresponding to different values of $\log I_0/I$, which are recorded on a single plate for a given solution, the total illumination time did not exceed six minutes. In every case, however, as a necessary precaution, the first exposure was repeated after such a series of exposure had been obtained, to make certain that no change had taken place during the process.

In handling the chicken and rat blood, a technique identical with that of Suhrmann's was employed. The samples were obtained by heart puncture, using a sterile hypodermic needle, and mixed at once with sodium citrate solution (0.5 cc. of a 5 per cent solution to 5 cc. of blood) to prevent clotting. These mixtures were then centrifuged and the supernatant plasma plus citrate solution were discarded. The blood corpuscles were repeatedly washed with normal saline solution (0.85 per cent) and centrifuged to remove the last traces of plasma and citrate. After the final washing, one cubic centimeter of the corpuscles was suspended in 99 cc. of distilled water (this concentration will be designated as = 0.01) and allowed to hemolize. The usual precautions for the cleanliness and optical purity of the distilled water were observed and all solutions were kept on ice, (at most, about 5 hours), until needed for the absorption measurements, but were allowed to come to room temperature before being placed in the apparatus. The solutions were well shaken before pouring into the absorption cells in order to obtain the oxyhemoglobin spectrum.

Owing to the indefiniteness of the concentrations of the blood solutions as obtained in the above manner, the absorption curves are plotted in terms of α, the absorption constant, where $\alpha = \beta c = 1/d \log I_0/I$. β is the true molecular absorption coefficient and d, the thickness of cell, in this case equal to 1.00 cm.

Discussion of results. In the case of the blood samples from the various healthy and rachitic chickens, the absorption curves were neither identical nor did they exhibit any regular differences in intensity. Figure 1 shows only the middle section of the spectrum for four different animals on an enlarged scale in order to bring out more clearly the irregularities.

These curves have been made to coincide at $\nu = 2600\ mm^{-1}$ ($\lambda = 3846$A) by appropriate multiplication of the absorption constant.[3] It will be noted that there is no correlation between the relative positions of the curves, even in the region between $\nu = 2600$ and $3100\ mm^{-1}$, where there is some apparent regularity, and the degree of rickets as estimated by the usual methods. This erratic behavior can no doubt be attributed to the fact that the blood cell of the chicken is nucleated: upon hemolysis, a solution is obtained in which are suspended particles of quite different sizes and absorptive powers. Visual observations of the solutions corroborated this hypothesis, for some were decidedly turbid, while others were almost entirely clear. Then again large numbers of dark particles were present in some of the solutions and almost absent in others.

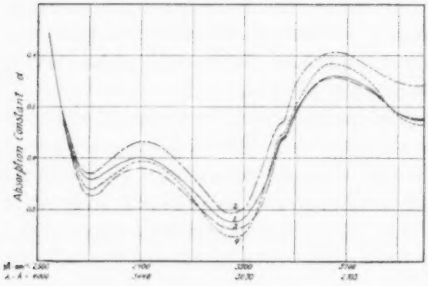

Fig. 1

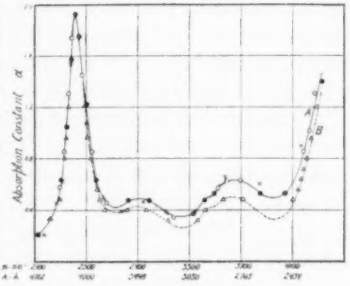

Fig. 2

Fig. 1. Absorption spectra of hemolyzed blood corpuscles from healthy and rachitic chickens: Curve *1*: Healthy. Curves *2* and *3*: 85 to 95 per cent cured of rachitis after irradiation with ultraviolet light. Curve *4*: Very rachitic.

Fig. 2. *A*. Absorption spectrum of hemolyzed blood corpuscles from healthy and rachitic rats: ● Healthy. ○ Rachitic. × Healthy (after Suhrmann). *B*. Absorption spectrum of hemolyzed blood corpuscles from healthy rat above after centrifugalization.

On the other hand, the rat bloods gave uniformly clear solutions, which could not be distinguished from each other and in which there was but a slight indication of finely divided suspended matter, in addition to the "blood ghosts," or stroma of the corpuscles. These latter were practically transparent and of sufficient size to settle out fairly rapidly. And now it was found that the absorption curves for the bloods of the healthy and the extremely rachitic animals were entirely identical. On figure 2, the full line curve, *A*, shows the absorption spectrum of blood from one pair of

[3] This multiplication is necessary because of slight concentration differences which are due to the method of making up the solutions. It is impossible to get the same number of blood corpuscles in one cubic centimeter because of the presence of slightly variable amounts of saline remaining behind after decanting from the centrifuge tube.

animals, the solid points being representative measurements (approximately one-third) from the data for the healthy animal and the hollow circles correspondingly for the rachitic one. This was checked for three healthy and three rachitic rats and all six curves, after appropriate correction for slight concentration differences (see footnote 3), can be exactly superposed with no variation greater than the limits of error in reading the plates. On the same curve, *A*, the crosses are selected points taken from Suhrmann's curve for his healthy rat and recalculated to the scale of our drawing. The over all agreement is generally good, with but a few scattered points lying irregularly above and below the curve obtained by us.

The curve given by Suhrmann for the blood of the rachitic rat falls above that for the normal animal and the separation between the two increases with apparent regularity toward the shorter wave lengths. In view of our experience with chicken blood where scattering of light due to suspended matter was a definite factor in producing discrepancies in the results for different animals, and also in view of the regular variation in the absorption spectra of the bloods of his healthy and rachitic animals, it seemed plausible to attribute this difference to the presence of different amounts of scattering matter in these solutions. The classical theory of light scattering was developed many years ago by Lord Rayleigh (1871); he was able to show that the intensity of the light scattered by particles, whose dimensions were small compared to the wave length of that light, was inversely proportional to the fourth power of the wave length and directly proportional to the concentration and length of path. If therefore a system, capable of absorbing light, also contains suspended matter, the total loss of light at a given wave length will be the sum of two factors, that due to selective absorption and another due to scattering. On this point of view, two solutions of the same substance which contained different amounts of suspended matter, should yield absorption curves having the same general form but differing in intensity. The more intense of the two will be for the solution containing the greater amount of suspended matter and its separation from the lower one will increase toward shorter wave lengths at a rate given by the inverse fourth power law: $\alpha = K/\lambda^4$. The application of this law to the two curves of Suhrmann yields a satisfactorily constant value for K.

It has also been found possible experimentally to duplicate Suhrmann's results in a qualitative manner and to confirm the part played by scattering in modifying the absorption spectrum. The hemolyzed blood solutions from a healthy and a rachitic rat were centrifuged to remove the ghosts and other suspended matter. The absorption spectra of these clarified solutions were then measured and found to agree perfectly between themselves; however, when compared with the absorption of the original unclarified solutions, the new curve fell below the former with the separation increasing toward shorter wave lengths. This is illustrated in

figure 2 by the broken curve, *B*, through the triangular points, which were obtained after centrifuging the original, hemolyzed blood solution of the healthy rat, used previously to obtain the full line curve. The difference between these two curves is due only to a difference in scattering and is in quantitative agreement with the Rayleigh inverse fourth power law.

The identity of the change of absorption produced by centrifugalization with that reported by Suhrmann for his healthy and rachitic rats seems to us to be definite proof that no chemical change of the hemoglobin has taken place because of the rachitic condition as suggested by him. This is, furthermore, completely substantiated by our finding that the absorption spectra of the bloods of healthy and rachitic rats are identical. The fact that his curve for the rachitic animals lies above that for his normal ones, which latter checks excellently with our curve for normal and rachitic, must therefore be interpreted as due to the presence of an excess of suspended matter in the former solutions. From the point of view of the physiology of blood, there is no apparent explanation for the occurrence of such an excess due to the rachitic condition of the animal. The fact that our results show no effect of this sort points to the probability that the change of absorption reported by Suhrmann and his collaborators was caused by some factor not necessarily associated with rickets. It is, of course, conceivable that the diet, by means of which his rats were made rachitic, may have been responsible for the nature of his observations. But in that case, the fact that we have not been able to reproduce his results with our normal and rachitic animals, raised here under standard conditions, obviously renders the method of blood absorption spectroscopy useless for general clinical practice.

SUMMARY

1. It has been shown that the absorption spectra of hemolized solutions of the red blood corpuscles of healthy and rachitic rats are entirely identical.

2. Experimental and theoretical evidence has been advanced to show that the change of absorption under these conditions reported by other investigators can be attributed to a scattering of light by suspended matter in the solutions and that no chemical change of the hemoglobin is involved.

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PATHWAY FOR VISCERAL AFFERENT IMPULSES FROM THE FORELIMB OF THE DOG

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It is generally accepted that sensory neurons exist in many of the internal organs such as the heart, lungs, kidney and intestines. When provided with an adequate stimulus they may conduct impulses interpreted as pain. Quite definite evidence has been presented to prove that the blood vessels, especially the arteries, also are supplied with sensory nerve fibers. Pagano (1900) showed that mechanical and chemical irritation of the intima of most arteries produces a rise in blood pressure. Spiegel and Wasserman (1926) showed that slight distention of the ascending aorta gave distinct reactions in experimental animals. Odermatt (1922) demonstrated that distention of arteries gives rise to pain reactions, and it has long been known that the injection of certain substances, such as barium chloride and lactic acid, into the arteries causes pain.

While it has not been conclusively proven that these afferent impulses are borne by neurons that differ in any essential way from somatic neurons, it seems probable that true visceral afferent neurons exist in peripheral parts of the body. While their course is unknown, it has been assumed that they travel with the cranial and spinal nerves (Friedrich, 1924), (Ranson, 1923), (Wiedhoff, 1924). In the case of a forelimb, it is assumed that the visceral afferents follow the mixed nerves to the cord; have their cells in the posterior ganglia, and enter the cord at the same level as do the somatic afferent fibers which accompany them in that particular mixed nerve. On the other hand, it is possible that there are afferent fibers in the gray rami communicantes. If this be true, it might be assumed that the visceral afferents follow a path similar to that of the vasomotor neurons to the forelimb, i.e., leave the mixed nerve by way of the gray rami, enter the sympathetic chain and from there reach the spinal cord through the white rami and posterior roots in the upper dorsal region. There are many clinical observations which seem to support this theory. Friedrich (1924) reports a case in which repeated amputations for the relief of pain had failed to alleviate the condition. Finally the somatic nerves near the elbow

were divided, but pain continued until sympathectomy was performed. One of us (W. K. L.) recently observed a case in which intractable pain in an arm was relieved by removal of the upper two thoracic sympathetic ganglia of that side.

It was to obtain evidence bearing upon these two opposing ideas that the present studies were undertaken. Preliminary tests showed that the injection of 5 per cent lactic acid into an artery in a lightly anesthetized animal causes an increase in blood pressure and depth of respiration. Even in rather deeply anesthetized animals this injection causes unmistakable evidences of afferent impulses. These consist of a rise in blood pressure and change in pulse rate together with increased respiration and a partial awakening from the anesthesia. An injection into the tissues of the thigh does not give such reactions.

To further check the use of lactic acid as an adequate stimulus for afferent impulses when injected into an artery we injected an ear artery of a large white rabbit. Immediately after the injection of 0.5 cc. of 5 per cent lactic acid the rabbit moved about so that we were unable to observe the ear color well. There seemed to be some vasoconstriction in this ear followed by a marked vasodilatation. When repeating this experiment on another rabbit similar results were obtained. On the basis of these observations it was assumed that the injection of 5 per cent lactic acid into arteries constituted an adequate stimulus for these afferent fibers. The fact that similar injections into muscle and fascia failed to give rise to a reaction, was interpreted as favoring the opinion that the reaction arose as a result of some effect on the artery itself rather than secondary effects on the somatic tissue.

The somatic afferents of the brachial plexus of a dog enter the cord from C5 to D2 inclusive. If visceral afferents may leave the mixed nerves by way of the gray rami communicantes to enter the sympathetic trunk and descend to enter the cord at a lower level, it might be possible that some of them enter the cord below D2. Therefore, injection of lactic acid into an artery of a forelimb which had all the posterior roots of the brachial plexus cut, *might* give rise to characteristic reactions. To obtain evidence on this point we operated upon a series of dogs to completely divide the posterior roots of the brachial plexus on one side. This operation can be performed with a relatively low mortality, and is more easily performed when young dogs are used.

Two dogs were permitted to recover for 8 to 9 days after deafferentation under full anesthesia. Then without anesthesia, all somatic sensory paths having been destroyed, the radial artery on the operated side was exposed. Injection of about 0.5 to 1 cc. of 5 per cent lactic acid produced no immediate reaction, but after a period varying from a minute to a minute and a half the animal gave unmistakable reactions of variable duration. This

was at first interpreted as being due to spasm of the vessels, the delay being attributed to the slow contraction of smooth muscle. A more logical explanation is that enough of the lactic acid reached the general circulation to affect arteries outside the field affected by the first operation. In both these dogs autopsy revealed the fact that deafferentation of the brachial plexus had been complete.

A third dog had had a section of the posterior roots of the brachial plexus (right side) 4 days previously. Under barbital anesthesia a cannula for recording blood pressure was placed in the carotid artery. Injection of salt solution under pressure was made into artery without producing appreciable change in blood pressure. Injection of 0.5 cc. of 5 per cent lactic acid made into brachial artery of operated side failed to give change in pressure. Similar injection into femoral artery gave prompt and marked change in blood pressure as revealed in figure 1. This recorded change was accompanied by marked changes in respiration. These injections were repeated and in each instance gave similar results.

In another experiment under barbital anesthesia an attempt was made to cut the dorsal roots from C6 to D2 inclusive on the right side. Both brachial arteries were exposed and a cannula for blood pressure tracing was placed in the carotid. Upon injection of lactic acid a positive response was obtained from each artery. However, autopsy revealed that the posterior half of the dorsal root of C8 had not been divided (fig. 2). An attempt was then made to check the possibility of this being the sole visceral afferent pathway from the vessel. Under conditions described above, another experiment was carried out whereby the posterior root of the 8th cervical nerve on the right side only, was cut. Lactic acid was then injected into each brachial artery with positive response from both. The 7th cervical and 1st dorsal nerve on the right side were then cut and the injection repeated. The response was less conclusive, perhaps because the arteries were in poor condition from the previous injection. Upon repeating this experiment the posterior roots of C7, C8, and D1 were cut on the right side. Injection of lactic acid upon the operated side now failed to give a typical reaction, while injection of the opposite side was strongly positive. These results were confirmed by second injections of each artery.

Under barbital-ether anesthesia a cannula was placed in the carotid artery of a dog for recording blood pressure, and both brachial arteries were exposed. The stellate and 2nd thoracic ganglia were removed on the right side by the trans-thoracic route. The thoracic cavity was then closed and 0.5 cc. of 5 per cent lactic acid injected into the right brachial artery. There resulted an almost immediate rise in blood pressure and an increase in the respiratory rate. Injection of the control artery gave almost an identical tracing. This experiment was repeated on another animal in

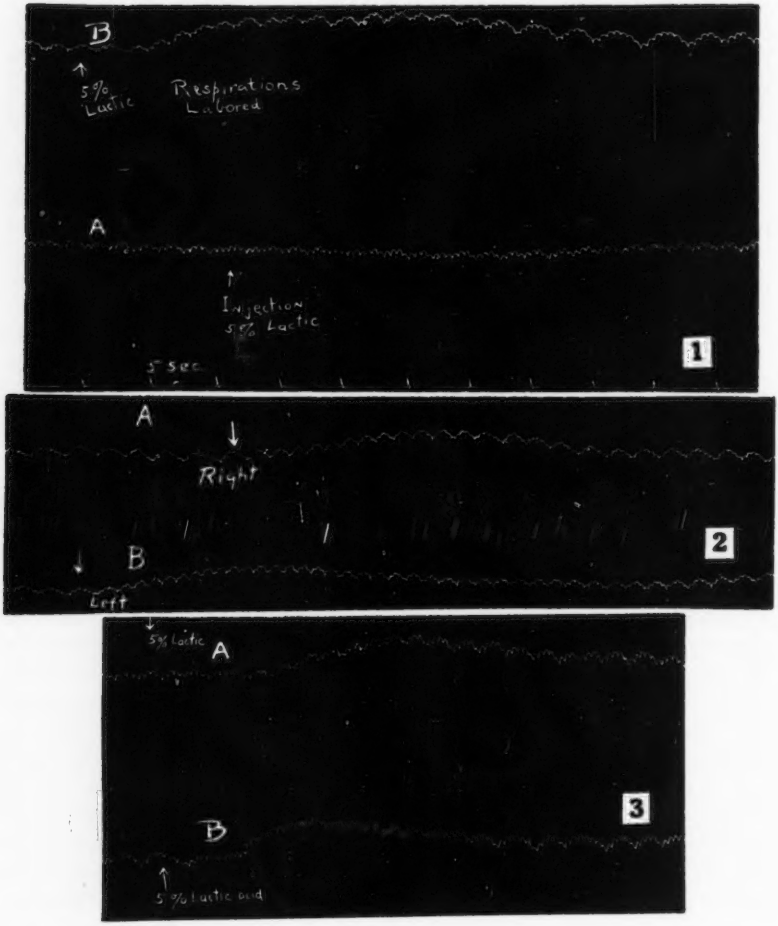

Fig. 1. *A* shows no effect upon blood pressure from injection of 0.5 cc. 5 per cent lactic acid into right brachial artery (deafferented limb), while *B* shows the effect of a similar injection into femoral artery of the same dog.

Fig. 2. Effect of 0.5 cc. of 5 per cent lactic acid injected into the brachial artery on right side following incomplete section of posterior roots of brachial plexus shown in *A*; *B*, the control injection on unoperated side.

Fig. 3. Effect of lactic acid injection into brachial artery after removal of stellate and second dorsal sympathetic ganglia shown in *A*; *B*, the effect of similar injection into brachial on unoperated side.

which the left stellate and 2nd thoracic ganglia were removed. Again the response on the operated side was similar to that obtained on the unoperated side (fig. 3).

These experiments confirm the experimental evidence of Dennig and tend to show that afferent impulses arising as a result of the injection of 5 per cent lactic acid into the arteries of the dog's forelimb travel to the cord by way of the mixed nerves and enter the cord in a manner similar to that of the somatic afferents through the posterior roots of the spinal nerves which they accompany. While not conclusive as to the exact upper and lower limits of these pathways our experiments seem to show that most, if not all of the afferent impulses resulting from this means of stimulation travel by way of the posterior roots of C7, C8, and D1 spinal nerves.

CONCLUSIONS

1. Injection of 5 per cent lactic acid into the brachial artery of a dog gives rise to afferent impulses which apparently are painful to the animal.
2. This reaction is dependent upon changes in the vessel wall rather than upon secondary changes in somatic tissues.
3. The removal of the stellate and second thoracic ganglia on one side does not alter the response of the animal to injection of lactic acid into the brachial artery of that side.
4. These visceral afferent impulses enter the cord largely by way of the posterior roots of the 7th and 8th cervical and 1st dorsal nerves.

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NERVE ACTIVITY AS MODIFIED BY TEMPERATURE CHANGES

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The various aspects of nerve activity as modified by temperature changes have been studied in this research to gain information concerning their interrelationships. Where the course of the change at various temperatures is the same, the assumption that the activities are controlled by the same factors is allowable but not proven. Where the behavior is different, the possibility of identical control is precluded, nevertheless from the degree of similarity some inferences may be drawn as to the extent to which the nerve functions in question may depend upon the same underlying processes.

THE TIME CONSTANTS OF THE SPIKE OF THE ACTION-POTENTIAL. The first question is whether the rising and falling phases of the action potential are prolonged to the same degree in cooled nerve.

Method. Unless special precautions are taken a comparison of the action-potential forms at two temperatures is subject to considerable possible error. This is largely due to the incremental nature of the start of the wave and the decremental nature of its ending. The crest of the spike is a point which can be marked without much difficulty, but the beginning and end of the wave are indefinite and therefore not readily located. For instance, if the wave of activity at a given temperature be recorded at different amplifications, different points for the start can readily be selected in the several curves simply because the first deviation from the base line is detectable earlier when the amplification is large; and for the same reason large amplification is apt to cause the end to be measured later. Similarly, when the wave is prolonged, as occurs in cold nerve, the less abrupt start and end lead to the same error even though the height of the spike be kept constant by adjustment of the amplification. In order to obviate this difficulty and also to eliminate any possible sources of error inherent in the irregularities of the coördinate system of the Braun tube on which the wave was recorded, a special procedure was adopted.

The stimuli were submaximal for the A wave, and the lead was taken

about 4 mm. from the stimulating cathode in order to obtain essentially the axon action potential form. As the end of the spike was a point of special interest particular precaution had to be taken to eliminate the diphasic artifact. This can be done successfully only when the potential change at the end of the nerve is nearly simultaneous with that at the active lead; the low potential at the end of the spike is then opposed by a low potential at the end of the activity at the dead-live junction. In order to obtain this condition, however, the nerve must be killed very close to the active lead and then there arises the danger of injury to the latter. This is a difficulty but not an insurmountable one, for with sufficient care or a sufficient number of trials it is possible to achieve the desired result. The nerve was killed as close as possible to the active lead, the spike potential being kept under observation to be sure that it was not thereby decreased; then the observations were made promptly before deterioration of the end of the nerve or partial reformation of a new plasma membrane could produce further complications.

The action potential was recorded at room temperature as a print of a single deflection on Duplitized X-ray Film, and also as a tracing on a sheet of cellophane. Following this the nerve was cooled, but before photographing the action potential, the coördinate system on the screen of the tube was adjusted so that the spike would have as nearly as possible the same dimensions as in the warm nerve. This was done by increasing the resistance in series with the condenser which controls the rate of the deflection along the abscissa, and by decreasing the shunting of the input to compensate the fall in potential.

All experiments recorded in this paper were performed on the sciatic nerve of the green frog.

Result. The method of recording is such that, if the spikes observed at the two temperatures have the same position on the abscissa and have the same shape when the film records are superimposed, the rate of change of potential in each part of the spike of the cooled nerve is shown to be decreased by a constant factor. The experiments were carried out in the range from 24° to 12°C., and are in complete agreement in showing that up to the time that the after-potential becomes visible, that is, up to the time that the potential has fallen below 2.5 to 5 per cent of the crest value, the records are completely superimposable. An example of two such records is shown in the upper part of figure 1.

Discussion. The constancy of the temperature coefficient for all parts of the spike negates some previous fragmentary observations; first, some experiments of Adrian's (1921) which are sometimes quoted in support of the idea that the falling phase has a larger coefficient than the rising phase, although no insistance was placed on the accuracy of the findings by the author himself; then, some data obtained by Bishop and Erlanger in the

course of an investigation of the effect of polarization on nerve activity, which purported to show the same thing. The latter experiment was confirmed by Gasser and Erlanger (quoted as a footnote in the reference cited);

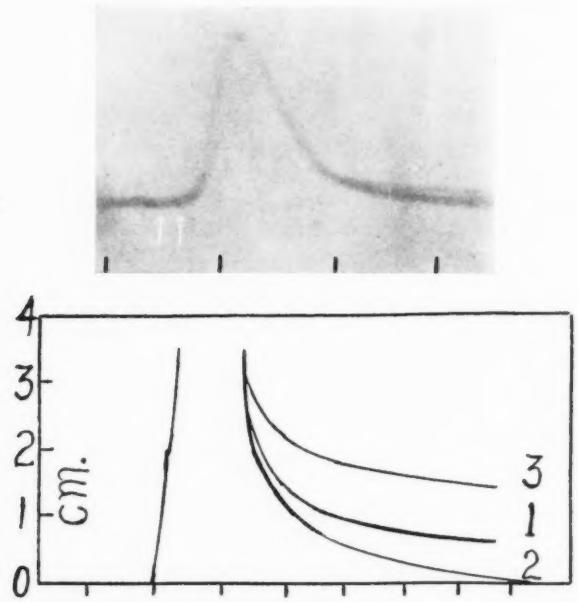


Fig. 1. *Upper part.* Action potentials recorded as single deflections at 14.2°C. and 20°C. The original film records were superimposed and printed on film. The reproduction is a print from the latter. The time intervals are 1σ for the record from the 20° nerve and 1.83σ for the 14.2° nerve. Conduction distance, 4 mm. Arrows show position of shock artifacts; left arrow for 20°C., right for 14.2°C.

Lower part. Tracing of the endings of three waves as recorded with four-panel amplification. *1*, nerve fresh, and at 21.6°C. *2*, nerve at 14.2°C. *3*, nerve at 20°C.; it now has developed a larger after-potential. The endings, *2* and *3*, correspond to the two spikes reproduced in the upper part of the figure. The time intervals are 1σ for curve *1*, 3.14σ for curve *2* and 1.71σ for curve *3*. Ordinates, deflection in centimeters; the amplification used for the records in the lower part is twenty-one times that used for those in the upper part. The notch on the up-stroke is the shock artifact, but the latter is of too short duration to interfere with the points under observation.

but on referring back to the original records it is now seen that a large part of the phase of falling potential had the same coefficient as the rising phase. The prolongation, which was at the end, can most probably be explained as a relative increase in temporal dispersion. The nerve was stimulated

with maximal induction shocks and there may well have been a visible displacement of the potentials in the slowest fibers in the course of the 2-3 mm. of conduction, which was permitted. The findings in this paper have been discussed with Professor Erlanger and Professor Bishop, and they are in agreement with the author that the former view should be retracted. Therefore the hypothesis that the rising and falling phases have separate underlying reactions loses its only support.

THE BEGINNING OF THE AFTER-POTENTIAL. In a cooled nerve the spike is prolonged, but in contrast to this the after-potential is shortened (Gasser and Erlanger). This means that where the action potentials at two temperatures are superimposed, as in the preceding section, there must finally come a time when the curves diverge. On account of the precautions taken to eliminate the distortion due to the diphasic artifact the nerves used in the preceding series were very favorable material for the determination of the first point of visible divergence. This was done as follows. After the spike-form had been photographed, another record was taken immediately in which the only change was an increase of amplification to four panels. In this way the divergence at the end of the spike was augmented without removing the possibility of calculating (with the aid of the amplification constant) the position of the point of divergence in the spike-curve.

Result. The deviation of the potential curves at the various temperatures began at about 1.5σ after the start of the wave as recorded at 22°C., at a time at which the potential had fallen to 2.5 to 5 per cent of that obtaining at the crest of the spike, the amount depending upon the magnitude of the after-potential. An example of such an observation is illustrated in the lower part of figure 1.

Discussion. The point where a constant temperature coefficient no longer obtains marks the time at which the after-potential first becomes visible; but whether or not it marks the real beginning of the latter cannot be stated definitely. A potential as low as the after-potential could not be detected on the steep slope of the falling phase of the spike, so the possibility remains that the condition responsible for it might start before the potential itself becomes evident. Similarly we have no means of knowing where the spike ends. It might be argued that doing the reverse of the present experiment, that is, following the temperature coefficient from the end of the after-potential forward, would make it possible to show the ending of the spike; but such a procedure would not be feasible. The after-potential is too labile to permit making the temperature the sole variable.

THE SPIKE AND CONDUCTION RATE. In the investigation of the spikes it was noted that the temperature coefficients were in the neighborhood of three, higher than the conduction velocities would be expected to be in the same temperature range. Also, as can be seen in figure 1, when the coördinate systems were adjusted so that the spikes were superimposable, the

distances from the shock artifacts to the starts of the spikes did not agree. They were relatively shorter in the cooled nerves, which meant that the conduction time (over the 3 to 4 mm. distance) was prolonged less than the duration of the spike. This lack of coincidence occasioned no little surprise as it not only did not fit the notion that the rising-phase of the action potential and the velocity are related (vide R. Lillie), but also ran counter to our own previous experience. The whole question therefore had to be reopened.

First the data collected for our previous paper (1928) were reëxamined. Two preparations had been used: 1, a nerve-motor-root preparation from the bullfrog; 2, a dog phrenic nerve with its sheath removed. In the frog preparation the axon-potential form had been recorded from the root with a lead 2 mm. from the stimulating cathode. The conduction distance was 3 mm. in the case of the phrenic nerve. In remeasuring the records the forms of the spikes were plotted, and their relative durations at two temperatures were determined by the factor which it was necessary to apply to the abscissa to make the spikes correspond. The remeasurement showed that, of the four frog-nerves cited, the conduction-time ratio corresponded with that for the spike duration in one case only; in two others the difference was definite; and in the fourth marked (the variable behavior is real for reasons which will be explained later). The correspondence between the rising and falling phases of the spike and the lack of correspondence with the conduction time indicate that a systematic error had been introduced into the previous method of measurement. The size of the shock artifact had made it impossible to locate the start of the action potential; therefore the rising phase had been measured from the artifact itself to the crest of the spike. This was the best method available at the time, and it was used in the belief that the small error included would not be significant. It now turns out however that the conduction time formed a relatively smaller fraction of the interval measured in the cooled nerves, and the error reached sufficient magnitude to lead to a misinterpretation.

In the phrenic nerve experiments the shock artifact was sufficiently small to permit measurement of the start of the rising phase; and the remeasurement of these records confirms the finding reported: the conduction time and the spike behave alike.

Method. The foregoing analysis indicates that a better method is needed for the measurement of the shape of the axon action potential, than the one involving a lead close to the stimulating cathode. Fortunately the need can be satisfied by a very simple and satisfactory technique. In place of leading from a point near the stimulus in order to eliminate temporal dispersion, the same end is attained by working with threshold fibers and sufficient amplification. The amplifier we are using at present gives about 130,000-fold voltage amplification, and it has been calculated that the equivalent spike height of the axon action potential of the A fibers as re-

corded at the stimulus is between three and four meters. A spike 1 cm. high would thus be one-third per cent or less of the total potential area. According to the principles enumerated by Gasser and Erlanger (1927) the number of fibers which would yield this potential may be estimated. In the paper cited they give a complete analysis of the ninth motor root of the bullfrog. The total area of all the fibers in this root is 180,000μ^2; 0.3 per cent of this amount is 540μ^2. As the threshold fibers are 20μ in diameter, one fiber on this basis would give a potential record about 8 mm. high. In a nerve trunk, where there are more nerve fibers, a single active fiber would produce relatively less effect due to the shunting by the inactive material; nevertheless it would take but a few fibers to produce a measurable wave. There is thus no chance for temporal dispersion; and in actual practice no prolongation is visible at any usable spike height at this amplification, even if conduction the length of the nerve be permitted. This method also has the advantage that there is no distortion of the beginning of the rising phase by the shock artifact.

The new method of recording the axon action-potential shape was first used to check the effect of temperature changes upon the parts of the spike. As before, the coördinate system was adjusted so as to make the records superimposable. The experiments were in complete confirmation of the former method.

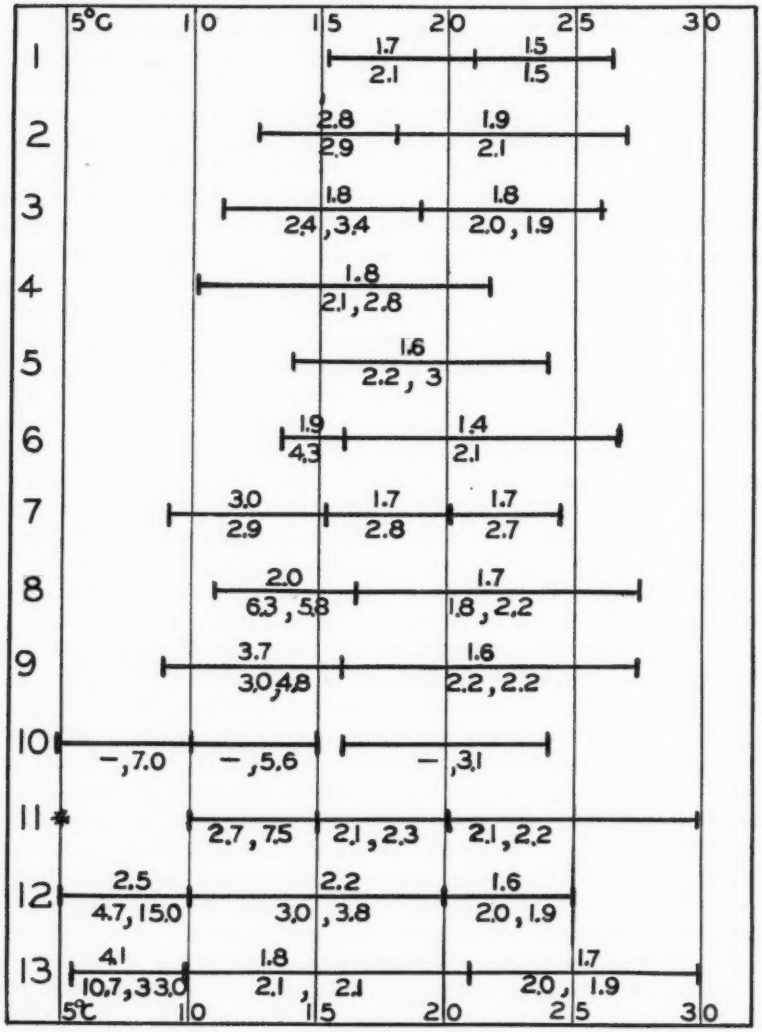
An investigation of the relation of the velocity to the spike was then undertaken. In part of the experiments short distances of conduction were used so that the data on the two points could be obtained from one record. In others separate records were taken for the conduction time and the spike, in order to get optimal conditions for each. The rising phase and the duration of the potential up to the time at which it rather abruptly starts upon its final gradual decline were measured to get the dimensions of the spike. Both measurements, within the limits of error, gave the same result when the temperature coefficients were calculated. The nerves were left at the temperatures at which the readings were made for about one-half hour, and were finally brought back to the initial temperature in order to eliminate the possibility of progressive change. It is evident from table 1 which contains the data and the calculated coefficients from these experiments, that a number of observations were made in the range between 25–30°C. The findings at these temperatures cannot be attributed to injury, as this was the temperature range in which the frogs were living and in which they displayed great activity. It was true however that there was greater impairment of conduction in the region of 5°C. than would be true for frogs accustomed to a colder environment.

Result. In many cases, particularly in the experiments performed in the early spring, the velocity and spike coefficients are very close together; but even here there is a constant deviation. The spike has a higher coefficient

TABLE 1

Temperature coefficients of nerve activities

The intervals at which the data were obtained are indicated by vertical marks; the values are calculated for 10°C. (Q_{10}). The value given above the line is for the conduction velocity, that below the line is for the spike. Where the latter is followed by a second value, the coefficient for the absolutely refractory period is given.



* This nerve gave a few responses at 5°C., then failed to respond altogether. On warming it made a perfect recovery.

than the velocity, and the difference at times becomes decided. The proximity of the two coefficients indicates an intimate relationship, but the deviation renders the conclusion necessary that the relationship is not the simple linear one which has previously been supposed.

REFRACTORY PERIOD. The effect of temperature on the refractory phase was determined in order to obtain data on nerves whose spike forms and conduction velocities were known. Adrian (1914) has shown that all stages of the recovery process are affected alike by temperature changes, and as far as we have investigated this point we have found no exception to this finding. Therefore, a measurement of the period of absolute refractoriness gives a determining value for the whole process. Stimulation was effected by break induction shocks. The coil used for the second shock was adjusted at each temperature to give a shock of just threshold value; then the resistance in the primary circuit was altered so as to multiply the shock strength by five. It was found by trial that such a shock gave a point on the recovery curve where the interval of response is no longer shortened by increasing the magnitude of the stimulus; and it was thus also proven that the error was avoided to which attention has been drawn by Forbes, Ray and Griffith: namely, the one arising from a prolonged utilization period of too strong shocks.

Result. The temperature coefficient for the refractory phase has a value clearly much closer to that of the spike than to that of the velocity of conduction. It is not identical with the former however, but tends to be higher. Little difference is encountered at the warmer temperatures; but as the nerve is cooled the coefficients increase to an unequal extent, that of the refractory period being greater, with the final result that at the lowest temperatures the refractory period shows a gross relative prolongation. In the case of some frogs which had become acclimated to a summer temperature of 30°C., or higher, conduction stopped altogether after a few stimuli at 5°C.,—in a perfectly reversible manner, however, as recovery was complete when the nerves were rewarmed.

These experiments are in accord with some previously reported by Adrian (1921), which showed the falling phase of the spike to have a coefficient close to that of the return of excitability, the latter coefficient being slightly higher. The increase which we found in the coefficient as the temperature falls is also paralleled in the one case where observations were made at two temperature intervals on the same nerve. Recently Amberson has published a temperature curve of the absolutely refractory period in which a Q_{10} of 3 applies throughout. The constancy of this coefficient is so at variance with our own experience that it is necessary to examine the data themselves. They are obtained under conditions which should give true values, but are presented as a composite curve which does not reveal the course of events in any one preparation. Also the observations stop at

10°C., at which point the greatest increase in the coefficient would be expected to begin in his frogs; in fact, they are in good agreement with our own experiments 9, 11 and 13 for temperatures above 10°C. Therefore we feel that this series of observations does not preclude the possibility of an increase of the coefficient at lower temperatures, or at higher temperatures in other nerves. The coefficients for all functions increase as the temperature falls, and one would hardly expect the recovery process to be an exception.

THE MAGNITUDE OF THE AXON SPIKE POTENTIAL. This does not seem to have been measured heretofore under conditions which would eliminate the effects of temporal dispersion. Where the absolute value of the spike potential is to be measured it is necessary for all the fibers to be stimulated in order that the number of fibers responding does not enter into the production of whatever changes are induced by the experimental procedure. This condition renders inapplicable the method of obtaining the action-potential form by recording, after high amplification, the conducted wave in threshold fibers, and makes it necessary to revert to the method of leading close to the stimulus. Also, changing the temperature of a nerve alters its resistance. Thus the potential drop across a recording device would be changed even though the axon potential remain constant. The resistance of the amplifier input is so high that an error of at most a few per cent is incurred even if no correction for the nerve resistance be made. On the other hand, because of their smaller value, input shunts cannot be employed for the control of the magnitude of the amplified potential. To meet the latter need a potentiometer was inserted between the second and third panels of the amplifier, and adjusted so that a maximal nerve response obtained at the upper end of the temperature range would have an appropriate height. The adjustment was maintained throughout the experiment; and, since the action potential is decreased by cooling, the arrangement gave assurance that all the spikes to be encountered during the experiment would be on the screen of the oscillograph and be directly comparable among themselves.

Since the measured potential, as an index to the axon-potential magnitude, presupposes that all fibers are active, a control experiment was performed to prove that the decrease in potential in cooled nerve is not due to a failure of some fibers to respond altogether. This was done by causing the nerve impulses to pass through a segment of the nerve surrounded by a cooling tube, and then recording them at the distal end of the nerve maintained at a constant temperature. The experiment showed that although the impulses were delayed and dispersed in the cooled region, the area recorded distally was constant. Thus activity of all fibers within the cooling tube was assured, and the method for obtaining the axon potential was validated.

Result. In every experiment cooling of the nerve caused the action po-

tential to decrease in a very characteristic way. Between 30° and 20°C. the potential falls slowly; then the rate of fall progressively increases, and somewhere in the neighborhood of 10°C. the decline becomes very rapid, especially in nerves from frogs accustomed to hot summer weather, where failure to respond altogether may occur around 5°C. Variations occur from nerve to nerve as to the abruptness of the change from a slow decline to a rapid decline, and as to the exact temperature at which the change occurs; but the same general form of curve obtains with great regularity as can be seen in the cases presented in figure 2.

RELATIONSHIP. A final series of experiments was performed in which the functions previously considered in groups of two or three were collectively followed in single preparations. Two of these are reproduced (fig.

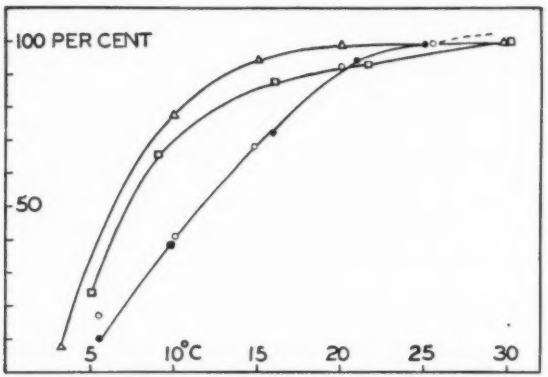


Fig. 2. Effect of temperature on the axon spike potential. Values recorded in percentages of those observed at the highest temperature examined. □, △—June frogs ●, ○—October frogs.

3), with the logarithm of the function plotted against the reciprocal of the absolute temperature. In this method of plotting as long as the temperature coefficient is constant the points can be connected by a straight line; and small deviations from linearity are less obvious than in the method used in figure 2. The method thus tends to bring out likenesses rather than differences. One of the two cases selected illustrates a close correspondence between the spike constants and conduction velocity, throughout a considerable temperature range; in the other the slope of the line denoting conduction time deviates consistently from that of the crest time or duration of the spike. It is not intended that these curves should be used as evidence that there is a definite break at a certain temperature. The number of points is too few to permit such a conclusion; and furthermore taking the series as a whole, the change seems rather to be progressive.

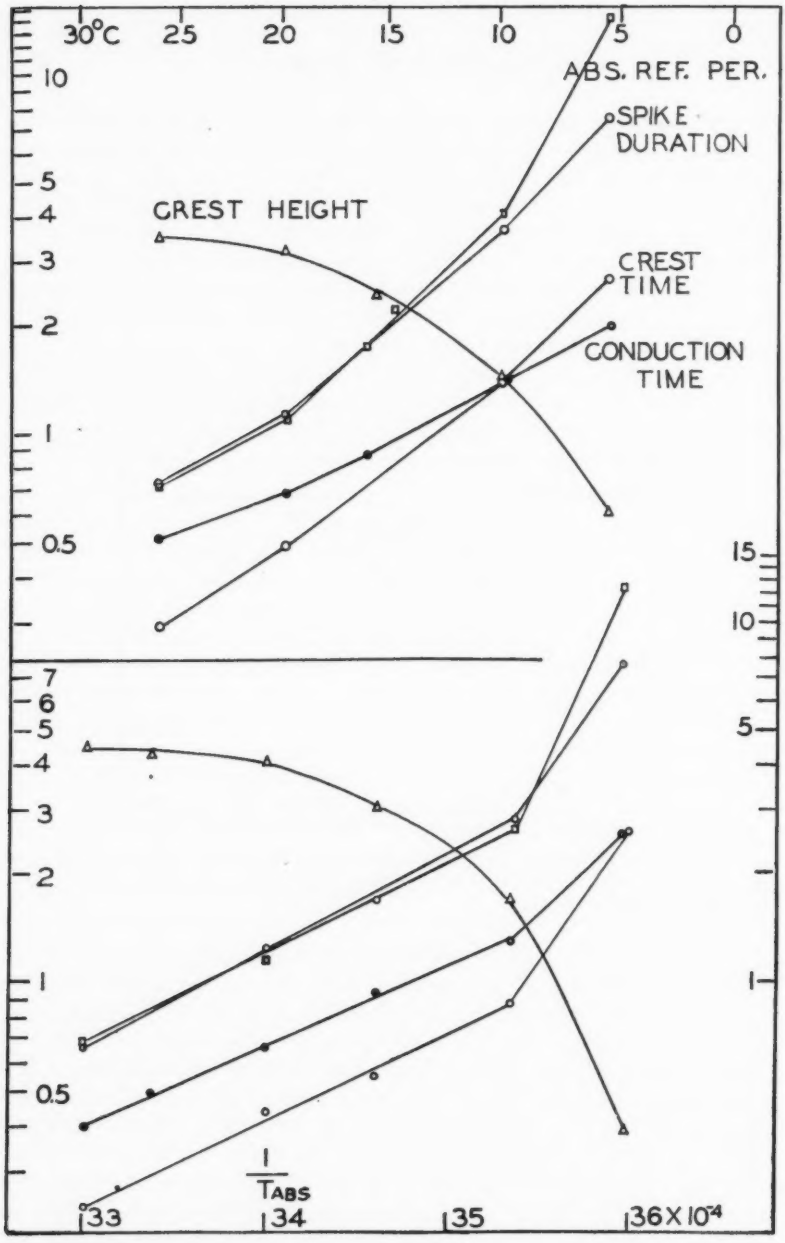


Fig. 3. Effect of temperature on nerve activities. The ordinates give time in sigmas and the measured crest height in centimeters.

The principal function not considered is chronaxie. A composite curve for the effect of temperature of the latter has just been published by Dworkin and Florkin; and it is interesting to note that this curve can be superimposed upon the crest-time curve in the lower group of curves in figure 3, for the points above 10°C. At 5°C. the chronaxie curve is lower, in that way resembling more closely the conduction time curve.

General discussion. The similarity of the slopes of the lines indicating the behavior of the spike constants, the velocity of conduction, the refractory period (and probably chronaxie) indicates the existence of some common controlling factor. On the other hand, the fact that each function in turn shows differences from the others proves that they are not identical in origin but depend upon their own peculiar set of conditions. Thus the values of coefficients obtained in experiments in all probability do not represent any single basic process, but are made up from a composite of processes. The behavior of the axon potential magnitude is much less closely related to that of the other qualities.

In searching for an explanation of the observations it must be clearly borne in mind that one can deduce the nature of a process neither from the temperature coefficient nor from the potential value. Chemical reactions in heterogeneous systems can have very low temperature coefficients; and physical qualities can be cited which have temperature coefficients covering the whole range possible for chemical reactions and extending beyond it.

If we may consider from the similarity of the slopes of the first parts of the temperature-activity lines that there is a common controlling process, then the temperature coefficient of this process has a value between 2 and 3. In the recent development of nerve physiology the relation of the nerve's metabolism to its activity has played an ever increasing rôle, and it is reasonable to suppose that the temperature coefficient of this metabolism would be a component in the determination of the coefficients as measured for the various functions. If this be the case the "2-3 component" of the coefficients would be accounted for as the coefficient of the underlying metabolism; but table 1 shows that there would still remain to be explained coefficients of conduction time ranging from 1.4 to 4.1, of spike duration ranging from 1.5 to 10.7, and of the refractory period from 1.9 to 33; and higher coefficients still could be calculated from short intervals in the range of the colder temperatures. Coefficients mounting to over 100 are to be encountered for the magnitude of the axon action potential.

A large variation of the temperature coefficient with the temperature interval under consideration is a generality in biological activities, as a reference to the instances collected in Kanitz' book will verify. As this variation is quite outside the range obtaining for homogeneous chemical reactions occurring in aqueous media, where the change is about 2 per cent

per degree centigrade, one is inclined to look about in the physical and chemical world for analogies. The only one found so far applies to the viscosity of such substances as fats, paraffins and lecithin. For instance, reference to a table of constants brought out the fact that the viscosity of rape oil increases 1.7 times between 30° and 20°C., 2.3 times between 20° and 10°, and 6.6 times between 10° and 0°; and it probably increases much faster in the next 10 degrees because the solidification point is −10°C. Paraffins in the appropriate temperature range also show the same rapid change. A mixture of paraffins was investigated by the author using the falling ball method of Gibson and Jacobs, with the result shown in the inset

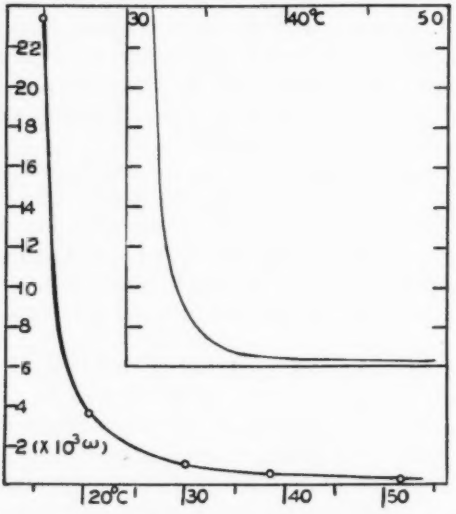


Fig. 4. Effect of temperature on the electrical conductivity of a solution of tetraethylammonium bromide in a mixture of alcohol and lecithin.

Inset. Viscosity of a paraffin mixture. Ordinate: Viscosity in arbitrary units.

in figure 4. As the viscosity of the medium changes, other properties of the system are known to change: for example, the electrical conductivity. To illustrate the latter point a 2 per cent solution of tetraethylammonium bromide was made in a mixture of lecithin (65 per cent) and absolute alcohol, and its resistance determined in a conductivity cell with a thousand cycle current and the cathode ray oscillograph with amplifier as a null instrument. Part of the resistance curve is shown in figure 4; to continue the curve to 10°C. it would be necessary to plot a point at 580,000 ohms.

While the finding of an analogy is not the production of an explanation,

it is not beyond the reach of possibility that the high temperature coefficients found in cold tissues may be due to characteristics of the surface film. Even monomolecular films have been shown by Adam to manifest viscosity, and if lipoids are concentrated in the surface in accordance with the theory they may be expected to increase their viscosity in such a way as to interfere with the development of the spike and after-potentials, and to prolong the recovery process responsible for the refractory phase. In the more normal range of activity the hypothetical viscosity factor would be expected to have but a small determining value on the course of events; but when it begins to mount rapidly it would markedly affect all functions. Then the slopes of all the curves would change radically; and that this is what happens can be seen in figures 2 and 3.

The idea that viscosity is a factor modifying nerve activity has been previously entertained. Sutherland considered it in connection with his physical theory, and Snyder used it to explain why the value of Q_{10}, interpreted as a chemical coefficient, should vary. The further allusions to viscosity which have been made in the literature of biological temperature coefficients have been recently reviewed by Bělehrádek, with the conclusion that the latter are really the temperature coefficients of the viscosity of the "reacting phases."

Nothing that is known about the viscosity of the axon cytoplasm indicates that it can explain the changes of the temperature coefficients of nerve. If the viscosity coefficient of the axon plasma is like that of blood plasma, then according to Snyder's data, it has a value like that of water; and the fact that the electrical conductivity of nerve changes like that of an aqueous solution of an electrolyte indicates that such is the case. This value is of far too small a magnitude to afford an explanation. Direct determinations of protoplasmic viscosity have been made by Heilbrunn on amoeba, using the rate of displacement of granules under gravity. The method probably measures factors which would not affect diffusion or conductivity, in analogy with the situation in a gelatin gel; and in any case the temperature curve obtained is very different from any of the temperature curves of nerve functions.

The different temperature curves obtained for the various nerve activities indicate that more than one process is involved; therefore viscosity of the medium does not afford a sufficient explanation of all phenomena. All nerves are not alike in their behavior and it is possible that the different controlling factors are operating to somewhat different degrees.

Of those considered, the lowest temperature coefficients for nerve are connected with the conduction time. Small values for conduction-time coefficients have been found quite generally by those who have investigated the upper temperature range, and these values cannot be attributed to injury; there is no forfeiting of reversibility. Conduction time and the

spike time-constants are closely correlated, but the frequently-found systematic deviation indicates some independence. Otherwise stated, the product of the velocity of transmission and the phase of rising potential is not a constant; but this need occasion no surprise as the velocity depends upon a number of factors other than the rate of development of the spike process (itself probably not a single entity).

If we picture the eddy currents through a point at any assigned distance ahead of the front of the wave we see that in a cooled nerve they will be decreased by the decrease in the axon-potential value and by the increase in the wave length (potential-distance), since the latter causes an additional decrease of the slope of the wave front. On the other hand, as explained in a previous paper (1928), the total energy of the current flowing through the point will be increased by the slower approach of the wave to the point. Excitation will depend not only on these considerations but upon the total energy necessary for the particular mode of application; therefore the analysis is blocked at this point because the latter value is one concerning which we have no information. What we do know from the end result is that the slowing of conduction occasioned by the change in the spike is more than compensated by other factors. Nothing is encountered which is prejudicial to the theory of excitation by local bioelectric circuits.

When considered in relation to the demarcation potential, the magnitude of the spike raises an interesting point. Bernstein showed the seat of the demarcation potential of muscle to be in the intact side of the fiber, because the potential is altered when the temperature of the latter is changed, but unaffected when the cut end is similarly treated. On repeating this experiment on nerve Verzár found that cooling the dead end was also effective, probably because there is some activity at the point from which the actual lead takes place. It therefore follows that significant values of the change of the resting potential are to be obtained only when the temperature alteration occurs under one of a pair of diphasic leads, or under the active electrode of a pair of monophasic ones. Verzár performed such experiments and found that when the nerve was cooled the potential was decreased 0.08 mv. per degree centigrade. This change is so slight compared with the decrease of the axon action potential that one can hardly consider as tenable the theory that the action wave, as a temporary local depolarization, has a potential determined by the resting value.

SUMMARY

The durations of the rising and falling phases of the spike of the action potential are affected to the same extent by temperature change.

The point at which the after-potential first becomes detectable was determined by making use of the fact that cooling prolongs the spike but

shortens the after-potential. This point, at 22°C., was 1.5σ after the start of the wave.

A simplified method of measuring the constants of the axon action potential is described.

While the temperature coefficients for conduction time and the duration of the spike are close together, a consistent difference between them appears; the former is smaller than the latter by an amount which varies somewhat from nerve to nerve. The product of the velocity and the duration of the rising phase is therefore not a constant; but this fact does not militate against the hypothesis that conduction is mediated through local bioelectric currents.

The temperature coefficent for the absolutely refractory phase is very close to that of the spike, but tends to be slightly larger, particularly in the lower temperature range.

The axon spike potential was measured at various temperatures. It declines in value along a characteristic curve which shows that the change is slow in the upper temperature range and very rapid in the lower one. A comparison of the data with those obtained by Verzár for the demarcation current indicates that the spike potential is not determined by the resting potential.

The temperature curves of the duration of the spike, refractory period and conduction velocity are sufficiently alike to suggest some controlling factor common to all three processes.

The magnitude of all the temperature coefficients increases greatly in the lower temperature range. This is particularly true in the case of the spike potential. An analogous behavior of the temperature coefficients of the viscosity of fats, paraffins and lecithin is described, and the suggestion made that the explanation of the high biological coefficients at low temperatures may be connected with viscosity of the plasma membrane.

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RESPONSE OF EXPLANTED EMBRYONIC CARDIAC TISSUE TO EPINEPHRINE AND ACETYLCHOLINE

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The opinion has become current in recent years that the effects which are produced from stimulating the vagus nerve are due to the elaboration of a substance having the properties of acetylcholine. Similarly a certain amount of evidence has been accumulated to show that stimulation of a sympathetic nerve results in the elaboration of an epinephrine-like body. These observations give a new aspect to the question of the locus of action of such drugs as epinephrine and acetylcholine. If it is the function of autonomic nerve endings to secrete these substances, then obviously their action must be more peripheral than the nerve ending. Whether these drugs can act by themselves on smooth muscle tissue or whether an intermediary receptive substance is necessary has not been determined. For this and other reasons I undertook to study the influence of these drugs on the hearts of chick embryos. The results obtained form the basis of this communication.

METHODS OF EXPERIMENTATION. In one series of experiments hearts from two ten-day-old chick embryos were excised and kept at room temperature in a covered Petri dish immersed in a carefully prepared Locke's solution without dextrose (Lewis, 1929). These hearts will be referred to as surviving embryonic hearts. No attempt was made to conduct these experiments in an aseptic fashion, although scrupulous cleanliness was observed and the solutions were sterile. After a little practice no difficulty was experienced in excising the hearts of chick embryos, except in the case of the two-day-old and three-day-old embryos when it was found necessary to employ a binocular dissecting microscope. Sharp instruments were essential.

In the other series of experiments the method of tissue culture as developed by Carrel and Maximow was employed. In brief this was as follows: The hearts were removed aseptically from embryonic chicks of various ages (two to ten days) and were cut into fragments of about 1 sq. mm. in area. These were explanted either on coverslips after the method of Maximow or in Carrel flasks type D. The flasks were found much more

suitable for this type of experiment because they permitted repeated addition of the drugs on successive days without contamination. The culture medium employed was a mixture of chick embryo extract and heparinized chicken plasma diluted with Tyrode's solution. The medium was changed every forty-eight to seventy-two hours, as indicated in the papers of Carrel and Maximow.

The fragment of embryonic heart that was explanted aseptically to this medium maintained its pulsations unimpaired and when examined three hours later showed regular vigorous contractions. Such explants were incubated in the usual manner at 39°C. When the effects of epinephrine and acetylcholine were to be noted the culture was removed to the stage of a microscope that was surrounded by a hot box kept at 39°C. When after a few minutes the rate of the pulsations became constant, the various solutions were added to it aseptically. Although bacterial contamination would, of course, occur, many cultures would be observed in this fashion on successive days without infection. This was particularly true when Carrel flasks were employed, since the necks and stoppers of these could be flamed.

It was assumed that the solution of epinephrine as purchased was sterile. The acetylcholine was made up before each test from the hydrobromide in sterile Tyrode's solution. The high acidity of the sample of acetylcholine hydrobromide precluded the chance of bacterial contamination. The acetylcholine was employed in a concentration of 1:1,000. The strength of epinephrine employed was as follows: 0.1 cc. of 1:1,000 epinephrine was added to 2 cc. of Tyrode's solution. Of these solutions 0.05 to 0.1 cc. was added to the culture whose total volume varied from 0.3 to 3 cc. depending on whether coverslips or flasks were used.

Surviving hearts were immersed in about 10 cc. of Locke's solution, and the same quantities as in the other experiments of epinephrine or acetylcholine were added in the immediate vicinity of the heart.

When deemed necessary the solutions were warmed to obviate changes in pulse rate due to changes in temperature. In each culture preliminary control experiments were performed by the use of Tyrode's solution in order to be sure that the few seconds during which the culture was at room temperature during the addition of the drug did not cause a significant change in rate, or that the Tyrode's solution itself would not upset the rhythm. Any culture that showed irregularities was discarded. A result was not considered positive unless following acetylcholine the heart stopped completely for thirty seconds and ultimately resumed its normal rhythm. In the case of epinephrine a result was not considered positive unless the rate accelerated at least 25 per cent, although usually the increase was much greater than this.

It may be stated that the pulse rates in any given fragment were unpre-

dictable but perfectly regular. Two fragments prepared from the same portion of one heart were often in close proximity in the same culture medium and yet they would beat at rates so different as 40 and 190 a minute. Although the rate varied from day to day it did not change appreciably over a period of hours during which the experiments were performed.

RESULTS. *Surviving hearts.* Almost invariably epinephrine and acetylcholine exerted their typical physiologic effects in the case of embryos that were six days old or more. In the case of younger embryos positive results became progressively fewer as the age of the embryo diminished. In the case of surviving hearts from five-day-old embryos the response was positive in more than 60 per cent. In four-day-old embryos it was more than 50 per cent. In surviving hearts prepared from three-day-old embryos, five hearts responded to acetylcholine and nine did not; six hearts responded to epinephrine, and sixteen did not. In the case of two-day-old embryos one responded to acetylcholine and fifteen did not; four responded to epinephrine and twelve did not. There was nothing to indicate from the appearance or the pulsation of a two-day-old embryo that anything was amiss with the technic, the isolation or the physiologic activity of the preparation. It must be concluded that a two-day-old embryo usually lacks something which is necessary in order that epinephrine and acetylcholine may manifest their typical effects.

Tissue cultures. These experiments differ from those on surviving hearts in that the tissue continued its growth, although it is probable that the growth is a poor approximation of the development of an intact embryo. For example, the fibroblasts proliferate much more rapidly than do the muscle cells and tend to outgrow the latter, although the muscle cells in most cultures also proliferate.

The explanted fragments responded to epinephrine and acetylcholine as did the surviving hearts. Almost invariably the fragments from six-day-old hearts or older ones gave positive results with epinephrine and acetylcholine. Moreover some of these tissue cultures pulsated for nine and ten days and at the end of that time still responded to the epinephrine and acetylcholine. As the age of the embryo diminished, the positive results became progressively fewer. With cultures from three-day-old hearts, sixteen fragments gave a positive response to acetylcholine and twenty-five did not; thirteen responded to the epinephrine and twenty-eight did not. In the case of two-day-old hearts, nine fragments responded to acetylcholine and twenty did not; twelve responded to epinephrine and eighteen did not. At first glance it appears that many more of the cultures from the two-day-old hearts responded than did the surviving hearts. If, however, the number of fragments responding to epinephrine and acetylcholine after four hours' incubation is considered it is seen that only one fragment of seven responded to acetylcholine and two in seven to

epinephrine. These results seem to indicate that the intermediary substance or cell that is necessary for the action of epinephrine or acetylcholine also proliferates. The following protocols show this in greater detail.

Two fragments from a two-day-old embryo were explanted in the usual manner. Four hours after explantation they were observed to pulsate vigorously and regularly at rates of 171 and 240 a minute respectively. The addition of epinephrine did not cause increase in rate. The next day the addition of epinephrine brought about its typical result. The pulsation increased from 100 and 160 respectively to 150 and 228 respectively. This result was obtained several times on different fragments. It is necessary to mention at this point that following such observation of the effect of epinephrine and acetylcholine on the tissue cultures these substances were washed away with Tyrode's solution before the culture was returned to the incubator.

In the case of acetylcholine results were similar. Several fragments were explanted from a five-day-old heart in the usual manner. Twenty-four hours later the effect of acetylcholine was studied; the rate in two fragments did not change whereas pulsation stopped for two minutes in the third fragment. The drug was washed away and the culture returned to the incubator for twenty-four hours. The influence of the drug was again studied and this time typical stoppage occurred in all three fragments varying in duration from one to three minutes. After the drug was washed away the normal rhythm was regained. Similar results are particularly numerous among the explants from two-day-old hearts which usually do not respond after three hours of incubation but after twenty-four to forty-eight hours the number of positive results is markedly increased. Here again growth for twenty-four hours or more results in the development of some intermediary body or substance that is essential for the action of acetylcholine.

COMMENT. The results show that at a certain stage in its development the embryonic chick heart pulsates regularly but is uninfluenced by such drugs as epinephrine and acetylcholine. Something appears to be missing in most seventy-two-hour embryos that is responsible for the typical effect of these drugs on the heart rate although direct observation of the pulsations of such embryonic cardiac tissue indicates nothing unusual. A consideration of this intermediary body or substance necessarily involves one in a consideration of the so-called receptive substance of Langley. My experiments do not indicate whether this receptive substance of Langley is an entity apart from the ganglion cells that develop in the heart independently of the outgrowth of the central nervous system or whether it is material that is not related to these ganglion cells. This substance can be cultivated in tissue culture and is very stable since cardiac tissue cultures that have been cultivated for ten days still give the typical reaction to epinephrine and acetylcholine.

These results were obtained in a study of 200 tissue cultures and ninety-four surviving hearts.

A word is necessary regarding what has been reported by anatomists concerning the development of ganglia within the heart muscle. Szantroch, in a study of the development of the intrinsic cardiac ganglion in the chick embryo, reported that ganglia were never observable before the seventy-second hour and usually appeared between the eightieth and the hundred fortieth hour of incubation.

My results suggest, therefore, that epinephrine and acetylcholine act on some substance which is distinct from the ganglia within the heart, and are offered as additional evidence for the existence of an intermediary receptive substance.

SUMMARY

A study was made of the influence of epinephrine and acetylcholine on the heart rate of chick embryos. The hearts were either excised and studied in Locke's solution or were explanted according to the methods of Carrel and Maximow. In general epinephrine and acetylcholine exerted their typical effect on embryos six days old or older. Many of the five-day-old embryos showed hearts refractory to epinephrine and acetylcholine and the action of these drugs became markedly less apparent as the age of the embryo decreased. In the case of seventy-two-hour embryos less than a third of the fragments responded.

Because of the lack of definite knowledge, both in regard to the exact site of action of these drugs, and to the time of development and beginning function of the intrinsic nerve mechanism of the heart, it would seem that these results can best be explained on the basis of some intermediary substance being necessary for the action of the drugs. In the case of explants from embryonic hearts it often happened that a fresh explant from a two-day-old heart would be negative to epinephrine or acetylcholine whereas on cultivation for twenty-four hours these drugs would exert their typical results.

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THE DIURETIC ACTION OF SECRETIN PREPARATIONS

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Several investigators (Launoy and Oechslin, 1913; Cow, 1914; Piticariu, 1916; Ambard and Schmidt, 1929; and Grinsberg, 1929) have observed diuresis to occur following the intravenous injection of secretin preparations and have ascribed considerable significance to this observation. Glaubach and Molitor (1928) found a diuretic substance in liver extract, which is claimed to contain a "pancreatic secretin." Dobreff (1925), working with plant extracts which he believed contained secretin, failed to obtain a diuretic effect. Since Drewyer and Ivy (1929) were unable to find secretin in plant extracts and in animal tissues other than intestinal and pyloric mucosa, since the extracts used by most of the investigators undoubtedly contained vasodilatin and were made by various methods, and since Mellanby (1928), using a highly concentrated secretin found no significant effect of secretin on the formation of urine, we decided to reinvestigate the claim that extracts of duodenal mucosa contain a diuretic substance. This, if true, might prove to be of considerable physiological significance.

Therefore, we have performed a number of experiments in an effort to answer the following questions: a. Do concentrated, vasodilatin-free preparations of secretin have a diuretic effect? b. Is the diuretic effect of secretin preparations species specific? c. Do extracts of various tissues prepared in a similar way to the secretin preparations, but containing no secretin, have a diuretic effect? d. Is the in vivo formation of secretin followed by diuresis? e. Is the twenty-four hour output of urine affected by the injection of secretin preparations?

Do concentrated, vasodilatin free preparations of secretin have a diuretic effect? A number of secretin preparations prepared in our laboratory were used, which may be briefly described as follows: a. Secretin preparation "A". This is the "new secretin" as prepared by the method of Weaver, Luckhardt and Koch (1926). This preparation actively stimulates pancreatic secretion in the dog in doses of 75 to 125 mgm. b. Secretin preparation "S I" which is water soluble, contains no vasodilatin, and stimulates pancreatic secretion in the dog in doses of less than one milligram. c. Secretin preparation "S II" which is soluble in 0.4 per cent HCl, contains no vasodilatin, and stimulates the pancreas of the dog in doses of less

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than one milligram. d. Secretin preparation "S III" which contains considerably more of the material called cholecystokinin than the other preparations and is free from vasodilatin. The details of the preparations and properties of these various extracts can be found in the paper by Ivy, Kloster, Drewyer and Lueth (1930). These preparations were made from the mucosa of the first six feet of the hog's intestine.

All these preparations were found to produce a slight but definite diuresis when injected intravenously into either anesthetized or unanesthetized dogs in amounts of five to ten times the minimal dose required to increase perceptibly pancreatic secretion. The anesthetized dogs were given sodium barbital (0.2 gram per kilo via stomach tube). The ureters were cannulated, connections were made for a blood pressure record, and the femoral vein was exposed under light ether anesthesia which was later removed. The unanesthetized dogs were prepared by suturing the trigone of the bladder to the abdominal wall as described by Dragstedt and Dragstedt (1928); the injections were made into the saphenous vein. All injections were made in less than 10 cc. of solution (usually 2–5 cc.), so that the fluid volume would be minimal. Following the injection of the secretin, there was observed first a latent period, usually from twenty to thirty minutes, occasionally longer, during which, in approximately one-half of the experiments, the rate of urine flow slightly declined, and second, a period of diuresis beginning twenty to thirty minutes after injection. The increased flow varied in degree from fifteen to one hundred per cent, and lasted from twenty to fifty minutes, always exceeding the slight decline that might occur during the latent period.

The following protocol is typical of the results obtained on ten dogs:

Male dog. Weight fourteen kilograms. 10:00 a.m., 2.8 grams sodium barbital given by mouth. 11:00 to 12:00, ether administered. Both ureters were cannulated, trachea cannula inserted, femoral vein exposed; left carotid artery connected for blood pressure record. Ether was withdrawn.

12:00–1:00.	Period allowed for recovery from the operation and effects of ether
1:00–1:10.	Urine flow 0.95 cc.
1:10–1:20.	Urine flow 0.95 cc.
1:20–1:30.	Urine flow 1.00 cc.
1:30.	10 mgm. secretin "S II" in 6 cc. water injected slowly into femoral vein.
1:30–1:40.	Urine flow 0.45 cc.
1:40–1:50.	Urine flow 0.45 cc.
1:50–2:00.	Urine flow 0.65 cc.
2:00–2:10.	Urine flow 1.30 cc.
2:10–2:20.	Urine flow 1.75 cc.
2:20–2:30.	Urine flow 2.80 cc.
2:30–2:40.	Urine flow 2.40 cc.
2:40–2:50.	Urine flow 1.00 cc.

Preparation "A" quite uniformly produced a greater diuresis (without a latent period) than the other preparations; this we demonstrated was due in part to its sodium chloride and hydrochloric acid content.

Is the diuretic effect of secretin preparations species specific? In the preceding experiments we have described the diuresis produced in dogs by the injection of secretin preparations obtained from hogs' intestinal mucosa. Secretin preparations have also been made from horses' and from dogs' intestines. These have shown the same type of diuretic effect on dogs so that it can be definitely stated that the effect is not species specific as reported by Piticariu (1916).

The following is typical protocol from results on ten dogs.

Female dog. Weight fourteen kilograms. 11:05, gave 2.8 grams of sodium barbital (Abbott's) via mouth. 1:00–2:00 p.m. Ether anesthesia. Ureters cannulated, femoral vein exposed, left carotid artery connected for blood pressure record. Tracheal cannula inserted. Ether removed.

2:00–3:30.	Period of recovery from operation and ether
3:30–3:40.	Urine flow 2.5 cc.
3:40–3:50.	Urine flow 2.3 cc.
3:50–4:00.	Urine flow 2.2 cc.
4:00.	200 mgm. secretin "A" from the horse, injected into femoral vein, solvent 8 cc. water.
4:00–4:10.	Urine flow 2.0 cc.
4:10–4:20.	Urine flow 2.6 cc.
4:20–4:30.	Urine flow 3.0 cc.
4:30–4:40.	Urine flow 3.1 cc.
4:40–4:50.	Urine flow 3.5 cc.
4:50–5:00.	Urine flow 3.4 cc.
5:00–5:10.	Urine flow 3.5 cc.
5:10–5:20.	Urine flow 3.2 cc.
5:20–5:30.	Urine flow 2.0 cc.
5:30–5:40.	Urine flow 2.1 cc.

Do extracts of various tissues prepared in a similar way to the secretin preparations, but containing no secretin, have a diuretic effect? Preparations from kidney, spleen, liver, colon, gall bladder and pancreas have been tested and found to have no diuretic effect. These preparations were made by the same technique used in obtaining secretin from the intestinal mucosa.

Is the in vivo formation of secretin followed by diuresis? Five female dogs were prepared with a bladder fistula, allowed to recover and the wound to heal after which they were again operated and a duodenal fistula made. In five other dogs a bladder fistula was made and then later a Thiry fistula of the duodenum. Weak hydrochloric acid applied to the Thiry fistula of the duodenum was effective in inducing diuresis as was the introduction of weak acid into the duodenum via a fistula. These experiments were controlled by the introduction of the same quantity of water, Ringer's

solution and saline solution. These solutions were used on different occasions. The two types of diuresis could be easily differentiated, since that resulting from the liquids occurred immediately and was not lasting, while the diuresis resulting when the weak acid was introduced showed two peaks, the first peak being due to the liquid only, the second peak to the delayed diuresis produced by secretin formed by the acid.

Further, in one of the Thiry fistula dogs, the Thiry fistula was surgically and chemically (phenol) denervated, and a diuresis resulted on the application of acid to the denervated loop. This diuresis was similar to that obtained by the formation of secretin in vivo.

Is the twenty-four hour output of urine affected by the injection of secretin preparations? For this study four female dogs were placed in cages. Arrangements were made to collect the urine. Water was constantly available. After suitable control periods secretin "S I" was injected. Three doses of 40 mgm. each in 10 cc. of water were slowly injected into the saphenous vein at intervals of four hours. The resulting urine samples varied little in amount from normal, a 10 to 15 per cent increase resulting as a rule. In no experiment did the secretin cause a decided increase in the twenty-four hour output of urine.

Discussion. Our results confirm the observations of other investigators cited above that an extract can be made of the upper intestinal mucosa which on intravenous injection causes a slight but definite increase in the rate of formation of urine. The active principle is not present in other tissues but is present only in those tissues which contain secretin, and as is true of secretin, it is not species specific. The active principle may be different from secretin, but we doubt it, since the diuretic activity of various preparations and concentrates parallels closely the secretin activity and in order to obtain a diuretic effect sufficient extract must be given to cause a copious secretion of pancreatic juice. Further, the fact that there is a long latent period (20 to 30 minutes) before diuresis results, which is also true when the in vivo formation of secretin is excited by introduction of weak acid into the duodenum, strongly suggests that the diuresis is secondary to the secretion of pancreatic juice and not due to the direct action of a specific substance on the kidney. This is analogous to the indirect or secondary effect of secretin on bile formation, in which there is a long latent period before an increased bile flow occurs (Mellanby, 1928; Lueth and Kloster, 1928) and that the exclusion of the pancreas prevents the increased bile flow.

The mechanism of the diuresis will be dealt with in a later paper by Dragstedt and Owen.

The failure of Dobreff (1925) to obtain a diuretic action from plant extracts which he claimed contained secretin, we believe was due to the fact that plant extracts which contain much vasodilatin when concentrated

according to the method used by us (Drewyer and Ivy, 1929) failed to contain secretin, and that the pancreatic secretion obtained by Dobreff was non-specific, was slight in amount, and due to the vasodilatin present (Ivy, 1930). We interpret Mellanby's (1928) failure as being due to the possibility that he did not inject sufficient secretin to cause a copious secretion; otherwise we must be dealing with a specific diuretic substance in our secretin concentrate, which seems doubtful to us. Further, Mellanby drained the pancreatic juice and bile to the outside, which is an important difference as will be shown in a later paper by Dragstedt and Owen. Our failure to obtain a diuretic substance from liver is contrary to the observation of Glaubach and Molitor (1928), but the method we used in preparing our extract was different.

Sakurai (1925) obtained a distinct increase in urinary flow on the introduction of 0.4 per cent HCl solution into the intestine, but interpreted his results as well as those of Piticariu as due to electrolytes in the solutions used. This possibility applies to one of our preparations of secretin but not the others which are known not to contain inorganic electrolytes. We do know, however, that the injection of a little sodium chloride solution enhances the diuretic effect of secretin.

CONCLUSIONS

1. "Purified" secretin preparations that are free from vasodilatin have a slight but definite diuretic effect.

2. The diuretic effect of secretin preparations is not species specific and closely parallels the secretin content.

3. Extracts of various tissues prepared in a similar manner to the secretin preparations, but containing no secretin, have no diuretic effect.

4. The in vivo formation of secretin is followed by a definite diuresis.

5. The twenty-four hour output of urine (dogs) is but slightly if at all increased by injections of secretin.

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ACHILLES AND CROSSED FLEXION REFLEX TIME IN THE INTACT RAT

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Allied reflexes have been subjected to a great number of investigations from the analytical approach in the last thirty years. However to our knowledge no one has studied these reflexes as they are integrated in the intact nervous system.

This paper reports the time relation between the Achilles reflex and the crossed flexion reflex as well as some conditions which change this relationship. The antagonistic drugs, caffeine and alcohol, were used to bring about this alteration.

APPARATUS. The apparatus has been described in detail elsewhere (5). Briefly it consists of a three stage resistance coupled amplifier, a portable three element Westinghouse Electric oscillograph, a vacuum tube oscillator and a signal circuit. The amplifier furnishes high amplification and as determined by controlled tests is exceptionally free from inherent jar. A supersensitive element capable of responding within 1/20,000 of a second was used for recording the electrical changes in muscles. The oscillator, a General Radio Company low frequency oscillator, type 377, was used to furnish a time line in units of 1/1000 of a second. The signal circuit which led to an oscillograph element was activated by discharging a condenser that had previously been charged.

The electrodes were thin brass strips, 3 mm. by 4 mm. covered with canton flannel and soaked, when used, in supersaturated salt solution. Both electrodes were set in a wooden block about 4 mm. apart.

The stimulus was given by a small metal hammer. When the tendon was struck the contact of the hammer with a small metal strip insulated from but directly over the Achilles tendon discharged the signal circuit. Although the stimulus was probably always maximal the intensity of the blow has been shown by Tuttle (6) to have no appreciable effect upon reflex time.

[1] The authors wish to acknowledge their indebtedness to Prof. Lee Edward Travis, in whose laboratory this work was carried on.

MATERIALS AND METHOD. The animals used were six mature male rats ranging in weight from 170 to 200 grams.

In order to elicit Achilles and crossed flexion reflexes each animal was placed in a split, hinged metal tube which covered the entire body with the exception of the head, fore and hind legs. Round the animal's neck was fastened a metal collar. The body covering and collar were made fast to metal stands which supported the animal about ten inches above the level of the work table. This arrangement immobilized the animal and left the hind quarters free for recording.

Achilles tendon reflex records were always taken from the left leg with the electrodes over the belly of the lateral head of the gastrocnemius muscle. Crossed flexion reflex records were always taken from the right leg with the electrodes over the belly of the tibialis anticus muscle. Both legs were shaved of hair to insure good contact.

TABLE 1

The effect of light and heavy dosages of caffeine and alcohol on Achilles and crossed flexion reflex time

RAT	NORMAL		0.5 MGM. CAFFEINE CITRATE PER 150 GR. BODY WEIGHT		2 MGM. CAFFEINE CITRATE PER 150 GR. BODY WEIGHT		LIGHT ALCOHOLIC INTOXICATION		HEAVY ALCOHOLIC INTOXICATION	
	A.R.*	C.F.R.†	A.R.	C.F.R.	A.R.	C.F.R.	A.R.	C.F.R.	A.R.	C.F.R.
R 1	6.5	9.2	8.1	9.2	6.2	7.6	6.0	9.5	6.5	10.0
R 2	6.1	9.0	7.0	9.1	6.5	7.7	5.5	9.5	6.6	None
R 3	6.5	9.7	7.1	9.6	6.5	7.8	5.7	10.0	6.7	10.5
L 1	6.5	10.1	7.2	10.4	6.6	8.9	6.0	10.0	7.0	10.5
L 2	6.2	10.2	6.9	10.0	6.5	9.0	5.5	10.0	7.7	12.0
L 3	6.3	9.2	7.1	9.2	6.5	7.7	5.5	9.2	7.5	None

* A.R. = Achilles reflex times in sigma.

† C.F.R. = crossed flexion reflex times in sigma.

Although the sequence of reflex elicitation was always, first left Achilles reflex and then crossed flexion reflex, preliminary experiments showed that varying the sequence had no effect on either reflex time. All times to be listed herein are the averages of from five to seven consecutive records.

All animals were subjected to the following experimental sequence. On a given day normal reflex time records were taken on both reflexes. On the following day 0.5 mgm. of caffeine citrate per 150 grams body weight was injected subcutaneously. Two days later 2 mgm. per 150 grams body weight of caffeine citrate were injected in the same manner. Reflex time records were taken from both reflexes fifteen to twenty minutes after each of the two injections. Following a period of a week the animals were placed in a bell jar in an atmosphere saturated with alcohol. For this paper an exposure to such an atmosphere for ten minutes constituted

light intoxication. Two days after the first administration of alcohol the animals were placed in the bell jar in a similar atmosphere for thirty minutes. This second exposure constituted deep intoxication. Reflex time records were taken on both reflexes immediately after the ten and thirty minute periods. During light intoxication no evident signs of depression were manifest but during heavy intoxication the animals were clearly depressed—some did not walk at all while others did so with the greatest difficulty.

The results obtained are presented in the accompanying table.

RESULTS. The injection of 0.5 mgm. of caffeine citrate per 150 grams body weight lengthened the Achilles reflex times but had no significant effect on the crossed flexion reflex times. A time change of 0.5 sigma or more in the rat was taken as significant since in no instance did consecutive reflex time records, taken at one testing, vary more than 0.5 sigma. Heavy dosages of caffeine gave Achilles reflex times similar to the normal but crossed reflex times were greatly shortened. Light dosages of alcohol shortened Achilles reflex times but did not significantly affect crossed reflex times. Heavy dosages of alcohol lengthened Achilles reflex times but to a lesser degree than crossed reflex times.

DISCUSSION. Workers from this laboratory (1), (2), (3), (4) have repeatedly shown that reflex times are longer than normal when taken under conditions which excite, and shorter than normal when taken under conditions which depress, the higher levels. In other words, any agent which excites the higher centers increases their normal inhibitory action on the passage of impulses over lower reflex arcs, and any agent which depresses the higher centers releases the lower arcs from the higher centers' inhibitory influence.

The action of mild dosages of caffeine increased the inhibitory action of higher upon lower centers to significantly lengthen the Achilles reflex time. It is to be noted that the inhibitory action of the higher centers was apparently not operating upon the crossed reflex arc since its reflex time was the same as normal. Heavy dosages of caffeine not only excited the higher levels but their effects spread to lower levels as well, so that the inhibitory action of the higher levels was in part offset by the facilitated lower levels with the result that a new equilibrium was established which expressed an Achilles reflex time about the same as normal. The crossed reflex arc again apparently was not under any inhibiting action as its reflex time was greatly shortened by this heavy dosage of caffeine.

The experiments with light alcoholic intoxication were the direct counterpart of those with small dosages of caffeine. Light dosages of alcohol depressed the higher centers thereby decreasing their inhibitory action upon the lower centers with the result that Achilles reflex times were shorter than normal. Under these conditions the crossed reflex times

were again not materially changed showing that a decrease in upper level activity has no effect on the speed of transmission over the crossed reflex arc.

The action of heavy dosages of alcohol did not parallel that of heavy dosages of caffeine. However, it is very difficult to control alcohol when it is administered as it was in this work. It was highly probable that the animals were depressed by heavy dosages of alcohol to a greater degree than they were stimulated by heavy dosages of caffeine.

In this series the alcoholic depression not only relieved the lower levels from the inhibitory action of higher levels but in some instances so greatly depressed the lower levels themselves that the resultant Achilles reflex time was considerable slower than normal.

The crossed reflex arc under these conditions was greatly depressed, with the result that crossed reflex times were considerably lengthened—in two cases the depression was so profound that no records were obtainable.

SUMMARY

1. Light dosages of caffeine citrate lengthened Achilles reflex times but did not effect crossed flexion reflex times.

2. Heavy dosages of caffeine citrate gave an Achilles reflex time similar to the normal but greatly shortened crossed flexion reflex time.

3. Light dosages of alcohol shortened Achilles reflex time but did not affect crossed flexion reflex time.

4. Heavy dosages of alcohol lengthened Achilles reflex time and also greatly lengthened crossed flexion reflex time.

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THE MECHANISM OF THE DIURETIC ACTION OF SECRETIN PREPARATIONS

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In the paper by Owen and Ivy a number of facts relative to the diuretic action of secretin preparations were brought out. These may be briefly summarized as follows: a. Purified, vasodilatin-free secretin prepararations, when injected intravenously into dogs produce a definite but moderate diuresis characterized by a latent period usually of twenty to thirty minutes; b, the diuresis is not species specific as secretin preparations from various sources are equally effective; c, tissue extracts prepared in a similar way but containing no secretin have no diuretic effect, and d, the in vivo formation of secretin following application of 0.4 per cent HCl to the duodenal mucosa will lead to a definite diuresis.

We were interested in the mechanism of the diuretic effect of secretin preparations for several reasons. In the first place the observation that secretin preparations produce diuresis has been used as an argument against the secretin hypothesis of pancreatic juice stimulation with reference to the specificity of the rôle of secretin. The merit of this argument would largely rest on the question of the mechanism of the diuretic effect for if the latter was indirectly produced it would not militate against the secretin theory, while if the diuresis was due to a specific local effect on the kidney, it might be considered antagonistic to the theory. In the second place, if the mechanism of the diuretic effect could be explained, such explanation might be of assistance in the analysis of the mode of action of other diuretic agents.

The first question that arose, therefore, in the analysis was whether or not any observable local effect on the kidney was demonstrable. Five dogs were anesthetized with barbital, the ureters cannulated, and oncometers adjusted to the kidneys for recording kidney volume changes. Vasodilatin-free secretin preparations[2] were injected intravenously, dissolved in 5 to 10 cc. of distilled water so that volume effect would be minimal.

[1] Josiah Macy Jr. Foundation Fellow.

[2] We are indebted to Ivy, Kloster, Drewyer and Lueth for the secretin preparations used in these experiments. A description of the preparations used can be found in their paper (1930).

Typical diuresis after a latent period of twenty to thirty minutes occurred in every instance without any perceptible change in the oncometer record. Such negative evidence might not necessarily preclude the possibility of local action on the kidney, but it led our investigation to other possibilities.

Cow (1914) had reported that a duodenal extract which caused a diuresis after a latent period of some thirty minutes, failed to do so if the pituitary gland was cauterized prior to injection. This led him to conclude that the duodenal extract excited the hypophysis to produce pituitrin, which in turn caused the diuresis. We attempted to verify this observation in the following experiments.

A series of five dogs was used. The animals were anesthetized with barbital, supplementary ether being used during the operative work when necessary. The hypophysis was extirpated through the mouth, cannulae inserted into the ureters and a femoral vein exposed. A period of one hour was then allowed for recovery from the operation and the effects of the ether. Purified vasodilatin-free secretin preparations (perceptibly active with regard to pancreatic secretion in doses of less than 1.0 mgm.) were then injected intravenously in doses of 5 to 10 mgm. per animal. Typical diuresis varying from a 20 per cent increase to a 100 per cent increase occurred in every instance, the latent periods varying from twenty to forty minutes. We were, therefore, unable to confirm Cow's finding that the integrity of the hypophysis is essential to the diuretic response.

A number of observations relative to the diuretic action of secretin preparations led us to suspect that the diuresis was indirectly produced. The comparatively long latent period after intravenous injection, and the absence of any oncometric indication of changes in kidney volume argued against the possibility of direct action on the kidney. It was further observed that for the most part the diuretic effect of a particular secretin preparation was proportional to its secretory stimulation of the pancreas. It therefore seemed probable that the diuretic effect was related to the secretory effect and might be dependent upon it. The following experiments were done to test this relationship:

1. The diuretic effect of secretin preparations after pancreatectomy and ligation of the common bile duct. Three dogs were anesthetized with barbital and supplementary ether during the operative work. The pancreas was completely extirpated, the common bile duct ligated, cannulae inserted into the ureters, and a femoral vein exposed. A period of one hour was then allowed to elapse for recovery from the operation and effects of ether. Several secretin preparations were then injected intravenously in doses shown to be adequate in other animals. In no instance was there any indication of a diuretic effect.

2. Dogs in whom the common bile duct has been cannulated, one pancreatic duct ligated and the other cannulated, the bile and pancreatic

juice being diverted to the outside, do not show any diuresis after the intravenous injection of secretin preparations. The introduction of the pancreatic juice and bile (that has been collected as the result of the secretin injection) into the jejunum is followed by the typical diuresis. A typical protocol illustrates this experiment.

Female dog; 10.2 kilogram weight. 2:15 p.m.—2.16 grams sodium barbital by mouth. 3:00–4:00 p.m. Added ether anesthesia. Cannulae inserted into common bile duct, major pancreatic duct, ureters and jejunum. Cannulae brought out through stab wounds and incisions closed. Ether stopped. 4:00–5:00 p.m. Period allowed for recovery from operation and effects of ether.

5:00–5:10. Urine flow 0.50 cc.
5:10–5:20. Urine flow 0.55 cc.
5:20–5:30. Urine flow 0.45 cc.
5:30. 10 mgm. secretin preparation "SI" into femoral vein
5:30–5:40. Urine flow 0.40 cc.
5:40–5:50. Urine flow 0.50 cc.
5:50–6:00. Urine flow 0.50 cc.
6:00–6:10. Urine flow 0.45 cc.
6:10–6:20. Urine flow 0.50 cc.
6:20–6:30. Urine flow 0.45 cc.
6:30. 10 cc. pancreatic juice plus 20 cc. bile collected between 5:30 and 6:00–injected into jejunum.
6:30–6:40. Urine flow 0.55 cc.
6:40–6:50. Urine flow 0.65 cc.
6:50–7:00. Urine flow 0.95 cc.
7:00–7:10. Urine flow 1.10 cc.
7:10–7:20. Urine flow 0.90 cc.
7:20–7:30. Urine flow 0.85 cc.
7:30–7:40. Urine flow 0.75 cc.
7:40–7:50. Urine flow 0.70 cc.
7:50–8:00. Urine flow 0.60 cc.

3. Dogs in whom a fistula of the duodenum has been prepared so that all of the digestive juices entering it can be diverted to the outside, do not show any diuresis after the intravenous injection of secretin preparations. The introduction of the fistula discharge (chiefly pancreatic juice and bile) collected as the result of the secretin stimulation, into the jejunum is followed by the typical diuresis. This experiment has been done in both the anesthetized and unanesthetized animals. In the latter case the duodenal fistula is prepared aseptically several days previously, and the trigone of the bladder sutured to the abdominal wall for collection of urine.

4. Inasmuch as the intravenous injection of secretin preparations causes an increased secretion of both pancreatic juice and bile, and a diuresis if both of these secretions are admitted to the small intestine, it was of interest to see if the diuretic effect was exclusively dependent on one or the other. Several experiments were performed to test this, of which the following is a typical protocol.

Male dog: 12.3 kilo. 1:00 p.m. 2.45 grams barbital by mouth. 2:00–3:00 p.m. Supplementary ether. Cannulae inserted into ureters, common bile duct, major pancreatic duct, terminal duodenum. Cannulae brought out through stab wounds, incisions closed, and ether stopped.

3:00–4:00.	Period allowed for recovery from operation and ether
4:00–4:10.	Urine flow 1.20 cc.
4:10–4:20.	Urine flow 1.10 cc.
4:20–4:30.	Urine flow 1.30 cc.
4:30.	15 mgm. secretin preparation "SI" intravenously
4:30–4:40.	Urine flow 1.35 cc.
4:40–4:50.	Urine flow 1.0 cc.
4:50–5:00.	Urine flow 0.9 cc.
5:00–5:10.	Urine flow 0.9 cc.
5:10–5:20.	Urine flow 0.9 cc.
5:20.	20 cc. of bile introduced through duodenal cannula
5:20–5:30.	Urine flow 0.95 cc.
5:30–5:40.	Urine flow 1.30 cc.
5:40–5:50.	Urine flow 1.25 cc.
5:50–6:00.	Urine flow 1.20 cc.
6:00–6:10.	Urine flow 1.00 cc.
6:10.	20 cc. pancreatic juice introduced through duodenal cannula
6:10–6:20.	Urine flow 1.00 cc.
6:20–6:30.	Urine flow 1.55 cc.
6:30–6:40.	Urine flow 1.70 cc.
6:40–6:50.	Urine flow 1.90 cc.
6:50–7:00.	Urine flow 2.00 cc.
7:00–7:10.	Urine flow 2.10 cc.
7:10–7:20.	Urine flow 1.8 cc.
7:20–7:30.	Urine flow 1.4 cc.

It is thus seen that bile has a slight effect in increasing the flow of urine, but that pancreatic juice is somewhat more effective. It is therefore probable that in the intact animal the secretin diuresis is dependent on both secretions entering the intestine, the relative effect of each depending in part on the amount of each formed as the result of the secretin injection, and in part on the fact that for equal volumes pancreatic juice is somewhat more effective than bile.

It seems quite apparent, then, that the diuretic effect of secretin preparations is dependent upon the secretion of pancreatic juice and probably bile, and the subsequent entrance of these secretions into the small intestine where they may be reabsorbed. It may be noted that in the experiments in which Mellanby (1928) reported that secretin had no diuretic effect, the pancreatic juice and bile were simultaneously collected by means of cannulae so that they could not enter the intestine. It is possible that the following events take place. The secretion of pancreatic juice and bile entails a slight withdrawal of fluid and electrolyte from the blood. The temporarily decreased rate of urine flow seen during the first five to twenty minutes after the injection of secretin is compatible with this view. This

abstraction of fluid and electrolyte from the blood is probably rapidly compensated from the tissues so that by the time the pancreatic juice and bile begin to be reabsorbed in the small intestine, equilibrium has been reached and the reabsorbed secretions are to a certain extent excreted.

CONCLUSIONS

The diuretic effect of secretin preparations is dependent upon their secretory stimulation of pancreatic juice and bile and only occurs when these secretions have access to the small intestine. Secretin diuresis is not dependent upon the integrity of the hypophysis or upon a local specific effect upon the kidney.

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THE GONAD STIMULATING AND THE LUTEINIZING HORMONES OF THE ANTERIOR LOBE OF THE HYPOPHESIS[1]

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The existence of a relationship between the anterior lobe of the pituitary gland and the ovaries of vertebrate animals has been definitely established. Facts which support this relationship are so well known that it is not deemed necessary to give a comprehensive review of the literature, since it may be found elsewhere (Fluhmann, 1929).

The evidence for the secretion of two distinct hormones by the anterior lobe of the hypophysis, namely, a growth hormone and a hormone functioning as a gonad stimulator, seems to be rather definite. On the other hand, while various authors have put forth the idea that there are two different hormones secreted by this gland, which act on the ovary, still no very definite evidence is at hand proving this contention. Zondek (1930a, b) gives indirect evidence of this idea, since his preparations from urine give two different pictures in the ovaries of rats, depending on the source of the urine. Evans and Simpson (1928) believe that there are two substances present which affect the ovary, but that one, namely, the luteinizing hormone is identical with the growth hormone. Claus (1931) offers good experimental evidence for the presence of two different substances in the anterior lobe which act on the ovary. However, as will be shown later, the substances which she obtained do not seem to be the same as those with which we are dealing.

In this paper we wish to present definite evidence for the presence of two distinct anterior lobe hormones which promote follicular and lutein development in the ovary. One of these is the gonad stimulating hormone which causes precocious sexual maturity when injected into immature rats. Its primary function seems to be the stimulation of follicular activity

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The desiccated anterior lobe powder which has been used in this investigation has been kindly furnished us by the Research Laboratories of Parke, Davis and Company.

in the ovary. The second is the luteinizing hormone which alone cannot affect the ovaries of an immature animal. It does, however, cause luteinization of the follicles which are produced by the gonad stimulator. These two hormones have been extracted quantitatively from dried pituitary glands and have been separated from each other in two fractions. Each of the two preparations produces a different effect from the original whole extract, but when they are again united a preparation is obtained which is entirely similar to the original. This fact is evidence that neither have been injured during the procedure.

GENERAL CHARACTERISTICS OF THE GONAD STIMULATING AND THE LUTEINIZING HORMONES OF THE ANTERIOR LOBE. We have not been able to extract the hormones, which stimulate follicular development and luteinization, from hypophyseal tissue by means of any pure neutral solvent. In order to obtain the active materials in solution, the gland must apparently be subjected to chemical treatment. However alkalies, such as sodium or potassium hydroxide, have an injurious effect on the gonad stimulating hormone and consequently cannot be used to advantage as extractives. Dilute aqueous solutions of ammonium hydroxide can be used but even here the yield of gonad stimulating hormone is decreased. On the other hand, the gonad stimulating hormone can be extracted by means of mildly acidulated solvents but in such solutions the luteinizing hormone is injured very readily so the resulting extract contains essentially the gonad stimulating hormone. Consequently none of these solvents can be used to advantage.

We have, however, found that aqueous pyridine will remove the two hormones from the gland without injury to either. An aqueous pyridine extract of the dried anterior lobe produces precocious sexual maturity together with tremendous luteinization of the ovary. When equivalent amounts of this extract and the emulsified anterior lobe powder are injected into sexually immature rats, the effect on the ovaries is approximately the same. This indicates that aqueous pyridine removes the hormones quantitatively and consequently this solvent has been used in the work which is reported in this paper.

The chemical characteristics of the two substances are somewhat different but their solubilities appear to be very similar. They are both soluble in acidified or alkaline aqueous solution with the difference, as noted above, that the gonad stimulating hormone is more stable to acids while the luteinizing hormone is more stable to alkalies. N/100 hydrochloric acid destroys the luteinizing hormone quite rapidly while at a pH of 4 its potency is impaired markedly. In relatively dilute solutions of strong alkalies, such as N/100 NaOH or KOH, the gonad stimulating hormone is destroyed quite readily. In one per cent NH_4OH it is not markedly injured for short periods of time while in fifty per cent aqueous

pyridine it is stable for several days. After standing for 60 days in such a solution, it was found to be completely inactivated. Both are insoluble in pure organic solvents such as ethyl ether, ethyl alcohol, petroleum ether, acetone or pyridine. The gonad stimulating hormone is quite readily soluble in cold distilled water while the luteinizing hormone is very slightly soluble. This difference in water solubility has been utilized as a means of separating the two hormones.

EXPERIMENTAL. *Preparation of the crude pyridine extract.* Five grams of dried anterior lobe are extracted for twelve hours with 200 cc. of 50 per cent aqueous pyridine. The extraction is carried out at room temperature in a test tube which has been fitted with a continuous stirrer. At the end of that time the extract is removed by centrifuging and the insoluble material is again extracted as before. The two extracts are united and evaporated to dryness in an air oven at 35°C. leaving a residue which may be emulsified in water and injected subcutaneously. This extract has the ability to produce sexual maturity in immature female rats, great follicular growth in the ovary together with numerous corpora lutea.

Separation of the two hormones. The dried pyridine extract is thoroughly triturated with freshly distilled water and the insoluble material is removed by centrifuging, reëxtracted and centrifuged. The water solution is evaporated to dryness in an air dryer and again taken up in water, centrifuging off all insoluble material. This procedure is repeated until the product is readily soluble in distilled water. The gonad stimulator is taken up in the water while most of the luteinizing hormone remains in the water insoluble fraction.

The aqueous solution is further purified by precipitation by means of alcohol. Five volumes of 99 per cent alcohol are added to the aqueous solution, causing the precipitation of the active material. The precipitate is centrifuged off, freed from alcohol and dissolved in distilled water as before. The aqueous solution is adjusted to pH of 4.0–5.0 and allowed to stand for 8 to 10 hours. A precipitate forms which is centrifuged off. The aqueous solution is neutralized to pH 6.8–7, precipitated by the addition of four volumes of alcohol, and dissolved in water.

The preparation which is obtained is water clear and can be prepared in a very concentrated form. It may be preserved by the addition of one volume of alcohol or by filtering through a Seitz filter. The latter procedure is scarcely to be recommended since the potency of the preparation is reduced due to adsorption. If the solution is made slightly acid before filtering the adsorption is decreased but still a marked loss of activity is observed. The absorbed material may, however, again be removed from the filter by washing with aqueous pyridine.

When this extract is injected into immature female rats they are brought

to sexual maturity in three days with production of large follicles in the ovaries with no or only slight indications of luteinization. This shows that the water soluble preparation contains primarily the gonad stimulating hormone with little or none of the luteinizing substance.

The water insoluble residue which contains the luteinizing hormone is washed with distilled water and taken up in N/200 sodium hydroxide. The solution is permitted to stand for 8 to 10 hours with occasional shaking. It is then centrifuged and the insoluble material discarded. The solution is neutralized and five volumes of alcohol are added. After the precipitate has separated out it is centrifuged, freed from alcohol and taken up in water at a pH of 7.6–7.8.

This preparation does not have the ability to bring an animal to sexual maturity but it does luteinize the follicles produced by the gonad stimulating

TABLE 1

Effect of pyridine extract of anterior lobe on the ovaries of immature rats. Equivalent of 0.02 gram dried pituitary glands injected daily for five days

ANIMAL NUMBER	VAGINA AT AUTOPSY	SMEAR	OVARIES	WEIGHT OF OVARIES		PER CENT INCREASE
				Experimental	Control	
				mgm.	*mgm.*	
1068	Open	Leucocytes	C.L. and F.	147.2	10.3	1,283
1098	Open	Leucocytes	C.L. and F.	144.9	12.4	1,069
1099	Open	Leucocytes	C.L. and F.	134.6	12.8	1,029
1100	Open	Leucocytes	C.L. and F.	128.1	8.7	1,372
1076	Open	Leucocytes	C.L. and F.	116.4	11.8	886
1078	Open	Leucocytes	C.L. and F.	121.5	11.8	1,215

C.L., corpora lutea; F, follicular growth.

fraction. If this fraction is united to the gonad stimulating preparation, and the two are injected simultaneously, the large luteinized ovary similar to that obtained by the use of the whole pyridine extract is produced. This fraction contains then the lutenizing hormone of the anterior lobe.

Methods of testing and results. The physiological activities of the anterior lobe fractions were determined by their effects on the reproductive tract of immature female rats, twenty to twenty-five days of age. The chief criteria used were the ability of the extracts to produce follicular and lutein development in the ovaries and opening of the vaginal orifice. Normally the vaginas of the rats in our colony do not open until they are about 60 days old and until this time the ovaries remain in a juvenile condition with very little follicular development and no corpora lutea. The anterior lobe extracts were injected subcutaneously in water solution, 0.25 cc. at a dose, twice daily for five days, the total equivalent of dried

anterior lobe for the entire period amounting to not more than 0.10 gram. At the beginning of the sixth day vaginal smears were taken, the animals killed and the ovaries weighed and fixed for histological study. Although we have used a large number of rats in the experimental work the results for a representative group of six rats injected with 0.1 gram of whole pyridine extract are presented in table 1. In the case of these rats the vaginas opened on the third day and on the fifth day the smears showed leucocytes. The weight of the ovaries had increased tremendously over that of the controls, showing an increase of 1000 to 1500 per cent. Macroscopically the ovaries appeared as masses of corpora lutea with few medium sized follicles, while microscopic study showed practically the same with the addition that many small follicles were present (fig. 2).

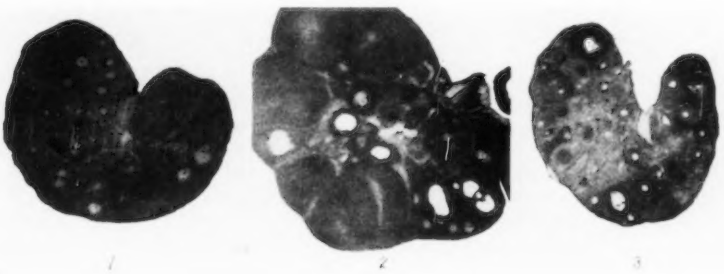

Fig. 1. Ovary of an immature rat. Twenty-eight days old.

Fig. 2. Ovary of a rat after five days treatment with whole pyridine extract of anterior lobe powder. Twenty-eight days old. Large corpora lutea and some follicles.

Fig. 3. Ovary of a rat after five days' treatment with water insoluble fraction of the aqueous pyridine extract of anterior lobe powder. Twenty-eight days old. Compare with figure 1.

The water soluble fraction of the pyridine extract produces quite a different result when injected in the same way in immature rats. In this case the rats' vaginas also open on the third day but on the fifth day the vaginal smears usually show a state of oestrus. The ovaries are much lighter as seen from table 2 and also contain primarily follicular growth. A microscopic study sometimes shows slight enlargement of the theca interna, indicative of stimulation to luteinizing activity and occasional corpora lutea. This may be due to the presence of traces of luteinizing hormone in the water soluble preparation. However, many of the ovaries show only follicular development (fig. 4). Table 2 gives the results from a representative group of six rats which have been injected with the equivalent of 0.10 gram of dried pituitary administered over a period of five days.

The second fraction, which has been freed from all water soluble material

and which is soluble in dilute alkalies does not have the property of bringing on precocious sexual maturity as evidenced by the opening of the vagina. The ovaries of immature animals, injected by this fraction, remain infantile, weigh the same as the controls and otherwise are entirely similar to those of the untreated animals (fig. 3). Table 3.

When the water soluble fraction is recombined with the water insoluble fraction we again obtain the fully luteinized ovary as in the original whole

TABLE 2

Effect of gonad stimulating hormone of the anterior lobe on ovaries of immature rats. Equivalent of 0.02 gram dried anterior lobe injected daily for five days

ANIMAL NUMBER	VAGINA AT AUTOPSY	SMEAR	OVARIES	WEIGHT OF OVARIES		PER CENT INCREASE
				Experimental	Control	
				mgm.	*mgm.*	
1045	Open	Oestrous	Follicles	30.0	13	131
1046	Open	Oestrous	Follicles	32.5	13	142
1047	Open	Oestrous	Follicles	43.9	13	237
1032	Open	Oestrous	Follicles	36.4	12	200
1037	Open	Oestrous	Follicles	28.2	11	156
1042	Open	Oestrous	Follicles	23.5	9.8	139

TABLE 3

Effect of luteinizing hormone of the anterior lobe on the ovaries of immature rats. Equivalent of 0.02 gram dried anterior lobe injected daily for five days

RAT NUMBER	VAGINA AT AUTOPSY	OVARIES	WEIGHT OF OVARIES		PER CENT INCREASE
			Experimental	Control	
			mgm.	*mgm.*	
873	Closed	Infantile	12.2	13.2	
872	Closed	Infantile	13.5	13.2	
852	Closed	Infantile	14.4	13.5	
856	Closed	Infantile	12.6	12.8	
864	Opened 5th day	Infantile	15.3	14.2	

pyridine extract, showing that the two active principles have been separated but not injured in any way. Table 4 gives the results of experiments carried out by separating the hormones as indicated, testing each fraction and also testing the united fractions. Rats 1234, 1246, 1232, and 1233 received the gonad stimulating hormone for five days. Numbers 1225, 1230, 1228 and 1229 received the luteinizing hormone while 1238, 1240 and 1241, received the united fractions for a similar period of time (fig. 5).

If the gonad stimulating hormone is injected for two days and the animals are killed on the fifth day the animal comes to sexual maturity on the third day as usual, but at autopsy the increase in the weight of the

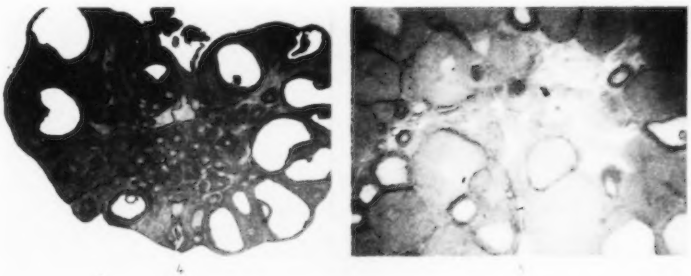

Fig. 4. Ovary of a rat after five days' treatment with water soluble fraction of the aqueous pyridine extract of anterior lobe powder. Twenty-seven days old. Large and numerous follicles with no lutein development.

Fig. 5. Ovary of a rat after treatment with the recombined fractions of the aqueous pyridine extract of anterior lobe powder. Twenty-eight days old. Large corpora lutea and follicles. Compare with figure 2.

TABLE 4

Effect of gonad stimulating, luteinizing hormone and combination of the two on the ovaries of immature rats. Equivalent of 0.02 gram dried anterior lobe powder injected daily for 5 days

RAT NUMBER	VAGINA AT AUTOPSY	SMEAR	OVARIES	WEIGHT OF OVARIES		PER CENT INCREASE
				Experimental	Control	
				mgm.	*mgm.*	
1234	Open	Oestrous	Follicles	21 2	9 8	116
1246	Open	Oestrous	Follicles	28 5	10 4	174
1232	Open	Oestrous	Follicles	31 6	9 8	212
1233	Open	Oestrous	Follicles	22 7	9 8	131
1225	Closed		Infantile	13 6	12 1	
1230	Closed		Infantile	16 3	14 3	
1228	Closed		Infantile	12 2	13 2	
1229	Closed		Infantile	13 5	13 2	
1238	Open	Leucocytes	C.L. and F.	117 5	10 7	996
1240	Open	Leucocytes	C.L. and F.	83 0	9 4	771
1241	Open	Leucocytes	C.L. and F.	104 0	9 8	963

ovary is very slight, and the anatomical changes in the ovary are likewise much less pronounced than if the injections are continued for five days. If, however, the injections of the gonad stimulating hormone are followed

by the luteinizing hormone for two days and the animals are killed at the end of five days, the ovary shows a marked change. Corpora lutea are present but the most noticeable change is the presence of numerous "blutpunkte." In some cases these are so numerous that the entire ovary is entirely red. The production of the "blutpunkte" described by Zondek evidently is caused by the action of the luteinizing hormone on the developing follicles. Table 5 gives the results for a representative group of rats which have been treated as stated above.

DISCUSSION. The experimental results which have been presented would seem to lead to only one conclusion, namely, that there are two separate hormones, elaborated by the anterior lobe of the hypophysis, which affect the ovary, one of which promotes follicular development and the other growth of the corpora lutea. The aqueous pyridine removes

TABLE 5

Effect of the injection of the equivalent of 0.05 gram of gonad stimulating hormone per day for two days followed by the equivalent of 0.05 gram luteinizing hormone per day for two days. Animals killed after 5 days

ANIMAL NUMBER	VAGINA AT AUTOPSY	SMEAR	OVARIES	WEIGHT OF OVARIES		PER CENT INCREASE
				Experimental	Control	
				mgm.	*mgm.*	
1384	Open	Leucocytes	B.P., C.L. and F.	58 3	10 8	439
1385	Open	Leucocytes	B.P., C.L. and F.	68 8	10 8	537
1386	Open	Leucocytes	C.L., B.P. and F.	31 8	10 8	194
1394	Open	Leucocytes	C.L., B.P. and F.	59 1	9 7	509
1391	Open	Leucocytes	B.P., C.L. and F.	40 2	9 7	314
1392	Open	Leucocytes	C.L., F. and B.P.	49 8	9 7	413

B.P., "blutpunkte"; C.L., lutein growth; F, follicular growth.

these hormones from the gland quantitatively since the residue after extraction is inactive while the extract is equally as active as the emulsified pituitary powder before extraction. We have, therefore, removed the active principles without injuring them in any way. The water soluble preparation contains all of the gonad stimulating hormone and is equally as potent in bringing about sexual maturity as is the whole pyridine extract. It contains at the most only small amounts of the luteinizing hormone. The luteinizing principle is present in the water insoluble but alkali soluble part of the pyridine extract associated with very little or none of the gonad stimulating hormone.

As a rule the luteinizing hormone cannot act on the ovaries of immature rats as is evidenced by the fact that no change occurs when such animals are treated with this fraction. However, a small corpus luteum or two may occasionally form in the ovaries of rats so treated, but the vaginas do not

open, the uterus remains infantile and there is no increase in the weight of the ovaries over those of the control animals. If the ovaries are first stimulated to follicular growth by means of the gonad stimulating hormone, and then treated with the luteinizing hormone, the follicles become luteinized and a typical mulberry ovary is produced. It is, therefore, a one-two reaction which must take place in the order which has been designated. Due to this fact it is possible to elicit the development of small follicles and opening of the vagina without the presence of corpora lutea by injecting small doses of the whole pyridine extract. However, if such treatment is continued or if a larger amount is administered corpora lutea invariably develop. The corpora lutea which are formed by the luteinizing hormone may be of two kinds, corpora lutea atretica and normal corpora lutea which follow ovulation. Usually both are found side by side. Often "blutpunkte" are present, similar to those described by Zondek when the urine of pregnant women is injected into immature mice and rats. These are caused by the capillaries of the theca rupturing into the follicular cavity. We have found that "blutpunkte" occur most often when the concentrations of the two hormones are very high. Particularly is this true if the gonad stimulator is injected first followed by a high concentration of the luteinizing hormone. Apparently the follicles are more readily converted into "blutpunkte" by strong action of the luteinizing hormone after they have attained a fair size. It is felt, therefore, that the rupture of the capillaries is due to the luteinizing hormone though more data are needed to prove this point.

The gonad stimulating hormone which we have obtained from the pituitary gland would seem to be identical with Zondek and Ascheim's Prolan A, which they have prepared from urine. The physiological and chemical properties which have been determined for the active material are the same in every respect. Prolan A produces only follicular growth with no luteinization when injected into immature rats or mice for two days and the animals are killed on the fifth day. This is also true when our preparation is used. However, if we continue the injections for five days and then kill the animals, we have found signs of lutein development in the ovaries of various animals. This is also true for Prolan A. This may be due to the presence of small amounts of the luteinizing hormone in our water soluble fraction or to some other factor such as the animal's own pituitary gland. This can be settled only by testing the extracts on the ovaries of hypophysectomized animals which is being done at the present time.

Collip (1930a, b, c,) has obtained a substance, Emmenin, from human placentae which has a stimulating action on the immature ovary. It produces sexual precocity associated with changes in the uterus and vagina characteristic of oestrus and in addition causes growth of follicles and

corpora lutea in the ovary. Chemically the substance which he has obtained is different from the anterior lobe hormones discussed here. His active material is soluble in acetone and 99 per cent alcohol, whereas the anterior lobe substances are insoluble in both. Also the placental hormone is relatively insoluble in water, while the gonad stimulating hormone of the anterior lobe is readily soluble. In a later paper Collip (1930d) reports that he has obtained a second fraction, insoluble in 85 per cent alcohol which also has a stimulating effect on the ovary. This fraction would seem to correspond more closely to the anterior lobe hormones.

It is also rather difficult to believe that the substances which were separated by Claus (1931) can be the same as the active materials with which we are dealing. Her active principles were extracted from dried anterior pituitary glands with acidified 95 per cent alcohol, and the luteinizing fraction was soluble in 99 per cent alcohol, whereas our active substances are both insoluble in these solvents. Using the same procedure Claus also obtained the gonad stimulating substance from a number of widely different sources, both animal and plant. It may be that she is dealing with a substance of a more general nature rather than a specific anterior lobe hormone.

As has been mentioned before, it seems that the tissue must be subjected to chemical treatment before the active substances are obtained in solution. In this connection a rather interesting fact was observed when a supply of dried glands was used which had suffered slight putrefaction during the drying process. The activity of the dried glands was not impaired but when we attempted to separate the hormones we were unable to do so. The luteinizing hormone was apparently as soluble in water as the gonad stimulator. This fact is mentioned here to emphasize the point that apparently the tissue must be well preserved in order to effect the separation of the two hormones by the methods which we have used.

SUMMARY

1. The anterior lobe of the hypophysis secretes two hormones which act on the ovary, a gonad stimulating factor which stimulates follicular growth and a luteinizing factor which causes lutein growth.

2. These two hormones have been extracted quantitatively from dried anterior lobe tissue by means of aqueous pyridine.

3. The two hormones have been separated from one another into two different preparations each of which gives a different physiological reaction. When the two fractions are again united the resulting preparation is entirely similar to the first crude extract.

4. The luteinizing hormone cannot act on the immature ovary. The infantile ovary must be stimulated to follicular activity by the gonad

stimulating hormone before a characteristic "mulberry" ovary can be produced.

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THE NATURE OF THE NERVE IMPULSE

II. The Effect of Cyanides upon Medullated Nerves

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It has been shown in a preceding paper (Schmitt, 1930) that carbon monoxide may effectively inhibit both the respiration and the propagation of the action potential in nerve, and that this effect is to a considerable extent reversible by exposure to light. The conclusion was drawn that nerve respiration requires a heavy metal catalyst similar to that postulated by Warburg, for the activation of molecular oxygen, and that the propagation of the electric action potential likewise requires activated oxygen provided by a similar catalyst. Whether or not the action potential is produced by the breakdown of substances thus oxidized or by a direct oxidation could not be determined directly. The experiments to be reported in the present paper parallel and corroborate the preceding experiments, cyanide being used as the heavy metal poisoner in the place of carbon monoxide.

1. The effect of cyanide on nerve metabolism. A. *Experiments at zero CO_2 tension.* The method employed for determining nerve respiration was similar to that previously described (Schmitt, 1930) except that one set of nerves was placed in cyanide solution, the companion set being placed in Ringer solution or phosphate buffer. Cyanide solutions were made up fresh for each experiment and were brought to a pH of 7.8. In cases in which strong solutions of cyanide were used the osmotic pressure of the solution bathing the control nerves was made equal to that of the cyanide solution by the addition of sodium chloride. Unless otherwise stated cyanide was used in the form of the sodium salt; when phosphate buffer was used this too was made up from sodium salts. The greater number of experiments were made at a temperature of 20 to 23°C. The nerves were from medium sized green frogs, R. pipiens.

From the preliminary experiments performed in this manner it became apparent that although they afforded some evidence of the inhibiting power of cyanides, the method was unsatisfactory for the reason that the curve representing the oxygen uptake of the cyanided nerves clearly

indicated an escape from inhibition after the first hour or so (see fig. 1). This result obtained whether the nerves were in Ringer solution or in isotonic buffer and was interpreted to mean that cyanide was escaping from the solution despite the fact that the pH was never lower than 7.8. This conclusion was borne out by the results of experiments in which the N/10 NaOH in the inset was replaced by N/10 or N/2 NaCN (unneutralized); in these experiments the inhibition remained constant from the first, amounting to 90 or 95 per cent in the case of N/100 NaCN (see fig. 2). If the nerves were treated with stronger solutions, e.g., N/7 NaCN, there was also no escape, the inhibition amounting to about 95 per cent over a period of hours.

To investigate further this escape phenomenon, two-bulbed vessels of the type described by Meyerhof and Schmitt (1929) were used, the manometers being of the differential type. For these experiments the nerves were placed in the right-hand vessel of each differential manometer. Then to one vessel were added 2.0 cc. Ringer solution or phosphate buffer and 2.0 cc. of the same solution containing cyanide to the other. Four-tenths cubic centimeter N/10 NaOH was placed in bulb *b* (see Meyerhof and Schmitt, 1929, p. 450) and 0.2 cc. of the same solution in which the nerves were suspended placed in bulb *a*. The left hand vessels served as control vessels and contained exactly the same solutions as the right hand vessels except that the volume of the nerves was made up by an equivalent amount of solution.

This method makes possible a closer analysis of the mechanism of the escape phenomenon described above. In the first place the large size of the vessels (volume = 19 cc.) permits the use of quantities of solution large enough for direct quantitative determination of cyanide concentration. Furthermore, by comparing the pH of the fluid in bulb *c* with that in the main chamber, *A*, information may be obtained as to whether the escape of HCN is due to diffusion out of the solution alone or to expulsion of HCN by the activity of the nerves, presumably by the formation of lactic acid. Similar information was obtained by comparing the escape of HCN from the fluid in the experimental vessel with that in the control vessels.

As may be seen from table 1, experiments performed under these conditions gave results quite different from those obtained with the smaller vessels. Little or no indication of escape from inhibition occurred over a period of hours in the case of relatively strong solutions, e.g., N/100 NaCN, (see fig. 3). In the case of weaker solutions escape occurred in from one to two hours and was never complete but became constant at a certain residual amount which varied with the strength of the cyanide solution. Figure 4 shows the effect of N/500 NaCN.

We next proceeded to determine the cause of this escape phenomenon.

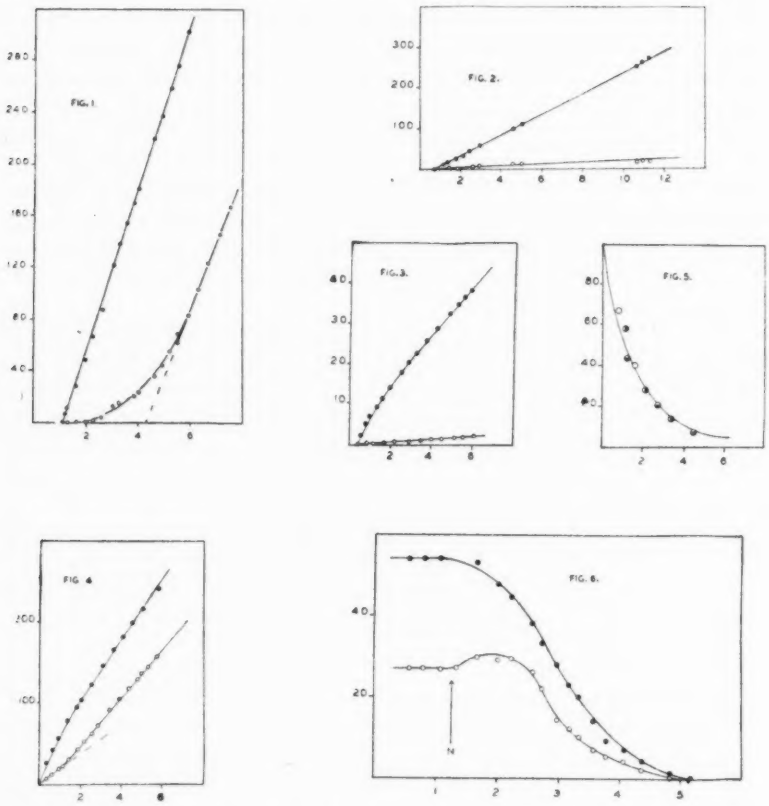

Fig. 1. Effect of N/50 NaCN on oxygen uptake. Nerves in small vessels, N/10 NaOH in inset. Circles represent oxygen uptake of experimental nerves; points that of control nerves. Ordinates represent cubic millimeters O_2 per gram nerve, wet weight; abscissae, time in hours.

Fig. 2. Effect of N/100 NaCN on nerve respiration when NaCN (unneutralized) is in inset instead of NaOH. Coördinates as before.

Fig. 3. Effect of N/100 NaCN on oxygen uptake. Nerves in 2.0 cc. solution in large vessels. Coördinates as before.

Fig. 4. Effect of N/500 NaCN on oxygen uptake. Phosphate buffer used instead of Ringer solution. Coördinates as before.

Fig. 5. Escape of HCN from solution by diffusion into alkali in inset. N/100 NaCN in phosphate buffer in large vessels; N/10 NaOH in side bulb. Ordinates, percentage of cyanide remaining in solution; abscissae, time in hours. Results of analyses at different time intervals from three separate experiments plotted together.

Fig. 6. Effect of methylene blue on failure of action potential in cyanided nerves. Companion nerves were dissected out. One was placed in buffered Ringer solution, the other in the same solution plus 0.002 per cent methylene blue; both were left in

That HCN diffuses out of the solution and is caught in the alkali in the inset was apparent from preliminary qualitative tests upon the alkali after several hours of shaking. To get some idea of the relative extent of this escape of HCN a method was devised for determining the concentration of cyanide in the Ringer or phosphate buffer solution from time to time under conditions similar to those of our experiments. In each of four two-bulbed vessels was placed 2.0 cc. of N/100 NaCN in phosphate buffer and 0.4 cc. N/10 NaOH in bulb *b*. The vessels were quickly attached to the manometers and shaken in the thermostat at 21°C. After a period of an hour a vessel was removed from one manometer, 1.5 cc. of the solution withdrawn, the vessel replaced, and the pair of vessels again placed in the thermostat for shaking. An analysis of the withdrawn fluid was then made according to the method of Viehoever and Johns (1916). In another hour or so another vessel was removed and 1.5 cc. of fluid withdrawn for analysis, and so on until fluid from all four vessels had been analyzed at different time intervals. The test is satisfactory for amounts of HCN less than 0.5 mgm. and will detect less than 0.01 mgm. Figure 5 shows the results of three separate experiments plotted together. From the curve it is evident that under the conditions of these experiments something like 70 per cent of the cyanide had escaped from the solution in two hours! The curve also demonstrates that the rate of escape of HCN from dilute solutions is much slower than from concentrated solutions, e.g., N/100 NaCN.

These facts make possible a more definite evaluation of preceding experiments in which alkali was used in the inset. There can be no doubt but that cyanide may almost completely inhibit nerve respiration whether the nerves are suspended in Ringer solution or in phosphate buffer. The escape from inhibition must be attributed largely to escape of HCN from the solution due to diffusion. It is also likely that the anaerobic formation of lactic acid plays a rôle in the phenomenon. Some significance may be attached to the fact that if the nerves escape from inhibition at all they do so in most cases between the first and third hours. According to Gerard and Meyerhof (1927), the anaerobic formation of lactic acid in nerves reaches a maximum in the second hour, thereafter falling off rapidly. According to Kolthoff and Furman (1926), the dissociation constant of lactic acid at 25°C. is 1.55×10^{-4} while that of HCN is 7.2×10^{-10}; hence

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the cold for 16 hours, to ensure good penetration of the dye. Nerves were then placed in hard rubber chamber so that the middle sections were submerged in the fluid: methylene blue Ringer solution on stained nerve, Ringer solution on unstained nerve. At *N*, NaCN was added to Ringer solution and to methylene blue Ringer solution to make concentration of N/1000. Points represent action potentials of unstained nerve; circles, those of stained nerves. Ordinates, deflection of action potential in millimeters on Braun tube; abscissae, time in hours.

TABLE 1

DATE	TEMPERATURE	CONCENTRATION OF NaCN	O_2 UPTAKE IN CU. MM PER GRAM PER HOUR		PER CENT INHIBITION	COMPOSITION OF FLUID	CONDITIONS AND RESULTS
			Control	Cyanide			
5/28/30	22.8	N/7	50.0	2.3	96	Ringer	4 cc. vessel with eccentric inset cont. 0.2 cc. N/10 NaOH. Nerves in 0.4 cc. Ringer or cyanide. No escape from inhibition
5/26/30	21.3	N/10	49.0	5.0	90	PO_4 buffer	Same. No escape
4/24/30	20.3	N/50	37.0	1.9	95	Ringer	Same. Escape in 1½ hours
5/21/30	22.1	N/50	58.0	4.0	93	PO_4 buffer	Same. Escape in 1½ hours
4/19/30	20.1	N/100	41.0	6.1	85	Ringer	Same. Escape in 2 hours
4/30/30	20.3	N/50 CH_3CN	44.0	43.0	2	Ringer	Same. No inhibition
4/ 9/30	18.3	N/100	26.1	2.6	90	Ringer	Same but N/10 NaCN in inset. No escape
10/16/30	22.7	N/100	35.2	2.2	94	Ringer	19 cc. vessels (2-bulbed). Nerves in 2.0 cc. fluid; 0.4 cc. N/10 NaOH in *b*; 0.2 cc. fluid in c. No escape
10/18/30	21.5	N/100	31.8	3.3	90	Ringer	Same. No escape
11/25/30	21.7	N/100	41.9	2.0	95	PO_4 buffer	Same. No escape
10/24/30	21.7	N/300	60.6	11.1	81	PO_4 buffer	Same. Escape in one hour to constant rate equal to 39 per cent inhibition
10/22/30	21.7	N/500	55.0	18.3	67	PO_4 buffer	Same. Escape in one hour to constant rate equal to 21 per cent inhibition
12/ 4/30	20.9	N/5,000	27.1	16.5	39	PO_4 buffer	Same. Escape in 2¼ hours to constant rate equal to 5 per cent inhibition
1/ 3/31	21.0	N/25	38.2	0.4	99	Bicarbonate	14 cc. vessels without insets. "Kästchen" method. Hamburger and Brinkmann Ringer. Gas: 5 per cent CO_2 and 95 per cent O_2
12/11/30	21.0	N/100	26.2	2.7	90	Bicarbonate	Same
12/22/30	21.0	N/100	29.8	4.1	87	Bicarbonate	Same
12/16/30	21.0	N/2,000	17.0	5.5	68	Bicarbonate	Same
12/29/30	20.9	N/10,000	44.8	28.3	37	Bicarbonate	Same

lactic acid formation may be a factor in the expulsion of HCN. Furthermore, according to Gerard and Meyerhof (1927) the lactic acid formed during anoxemia is not resynthesized or oxidized in oxygen. Hence any partial escape from inhibition due to acid expulsion of HCN from the inner nerve fibers will depend upon an equilibrium between the formation and diffusion outward of lactic acid and the diffusion inward of HCN (or NaCN). Apparently in experiments with two-bulbed vessels, N/100 NaCN solutions are sufficiently concentrated to push this equilibrium almost completely in the direction of free HCN despite the progressive dilution of the solution by diffusion, according to the curve in figure 5. Although these complicating factors make it impossible to determine accurately the inhibiting effect of cyanide in various concentrations, it may be said with certainty that cyanide is capable of inhibiting nerve metabolism practically completely (90–100 per cent) and that the degree of inhibition does depend, within limits, upon the concentration of the cyanide.

B. *Experiments at physiological CO_2 tension.* To eliminate the factor of escape of cyanide by diffusion into the alkali and to meet the objection of Warburg that the respiration of animal tissues is not normal under conditions of zero CO_2 tension, a number of experiments were performed using the "Kästchen" method of Warburg (1924).[1] Because of the low rate of oxygen consumption of the nerves and because of the great inhibition produced by cyanide relatively large amounts of tissue had to be placed in the vessel (about 150 mgm. wet weight). Experiments were carried on under a variety of conditions; both the bicarbonate concentration of the Ringer solution and the ratio of CO_2 to O_2 were varied in an attempt to obtain optimum experimental conditions. It was concluded from this preliminary investigation that the use of Ringer solution containing 0.285 per cent bicarbonate (Hamburger and Brinkmann, 1918) in a gas mixture containing 5 per cent CO_2 and 95 per cent O_2 was most satisfactory. The pH was 7.3 and nerve respiration under such conditions agreed well with the respiration of similar nerves measured with the alkali inset method.

Typical data obtained under such conditions are presented in table 1. N/25 NaCN caused complete inhibition; diluting the cyanide caused progressive decrease in the percentage inhibition, although N/10,000 NaCN still gave 37 per cent inhibition. The values obtained with this method agree very well with those obtained with the alkali inset method when allowance is made for the difference of pH in the two methods. Dixon and Elliott (1929), using mammalian brain, obtained from 80 to 91 per cent inhibition with N/30 KCN.

Organic cyanides, according to Warburg, (see Toda, 1926) have no

[1] We take this opportunity of expressing thanks to Prof. David Barr for the loan of the vessels and manometers used in this work.

inhibiting effect; only isonitriles are effective. This we find holds true also in the case of nerve. Even in M/50 solution, acetonitrile has no measurable inhibiting effect (see table 1).

Discussion. Considerable theoretical significance attaches to the question of the completeness of cyanide inhibition of respiration of animal tissues. If the postulates of Warburg are correct tissue respiration is mediated *entirely* by the heavy metal containing respiratory enzyme and hence, if cyanide is efficient in poisoning this enzyme, as Warburg claims, tissue respiration should be completely inhibited in the presence of cyanide. To test this point, Dixon and Elliott (1929) made a comprehensive investigation of the effect of cyanide in varying concentrations on the respiration of a number of typical animal tissues. They found that the maximum inhibition in the different tissues varies between 40 per cent and 90 per cent, the average value being 60 per cent. Furthermore, N/1000 cyanide usually produced maximum inhibition; increasing the concentration a hundred-fold had little effect. From this they concluded that the respiration of animal tissues is made up of two components: one part which is sensitive to and another part which is stable to cyanide. Barron (1930) also states that tissue respiration is not completely inhibited by cyanide though no data are given. Alt (1930), working under Warburg's direction tested the effect of cyanide upon the respiration of kidney, liver and spleen using the "kästchen" method and found that N/100 HCN sufficed to inhibit completely the respiration of these tissues. Decreasing the concentration of cyanide decreased the percentage inhibition as claimed by Warburg. The criticism levelled at the work of Dixon and Elliott was that their experiments were done under unphysiological conditions since the tissues were suspended in phosphate buffer and since the method was one involving zero CO_2 tension.

In discussing the bearing of the present experiments upon the controversy it should be stated at the outset that varying the ionic content of the Ringer solution has, within limits, little effect upon the percentage of maximum inhibition produceable by cyanide. The oxygen uptake of resting nerves from fall and winter frogs, expressed in cubic millimeters per gram wet weight per hour, averages 32 for bicarbonate Ringer (kästchen" method), 45 for phosphate buffer (alkali-inset method) and 34 for plain Ringer solution (alkali-inset method), while the maximum inhibition obtained with N/100 NaCN is 89 per cent, 93 per cent, and 88 per cent, respectively. Accordingly, the presence of CO_2 has little effect on the resting rate while phosphate buffer may increase it as much as 30 per cent.

Dixon and Elliott (1929) used cyanide in the form of the potassium salt but they do dot state whether the phosphate buffer was made from potassium or from sodium salts, although they do mention that the uptake in Ringer solution and in phosphate buffer were identical. In our experience,

nerve respiration may be greatly decreased by substituting KCl for the NaCl in Ringer solution; in one experiment the decrease amounted to as much as 56 per cent. In the case of rat liver the oxygen uptake of the control tissue in phosphate buffer reported by Dixon and Elliott was very considerably less than that given by Alt (1930) for the same tissue using bicarbonate buffer despite the fact that Dixon and Elliott were working at a somewhat higher temperature than Alt. If the control tissues were respiring at only a fraction of their normal rate it is possible that the inhibition percentages obtained for cyanide by Dixon and Elliott were all correspondingly too low.

Anent the present discussion of the degree to which respiration may be inhibited by poisoners of the respiratory ferment, Warburg and Kubowitz (1929) have shown that the respiration of yeast may be inhibited completely by mixtures of CO and O_2 provided conditions of gas diffusion and the relation of cell surface to cell mass are ideal. Such ideal conditions are not realizable in the case of the cells of animal tissues. In a similar manner, it is almost certain that the choice by Dixon and Elliott of cyanide as a poisoner to test the Warburg theory in the case of animal tissues was unfortunate. Among the conditions which complicate the situation may be mentioned: the escape of HCN from the solution into the alkali in the inset (a glance at figure 5 will show that this consideration is more important than Dixon and Elliott believe); the expulsion of HCN from the interior of the cell by lactic acid formed as a result of the anaerobiosis produced by the cyanide. Since lactic acid is stronger than HCN by some six orders, there can be no doubt but that this is an important factor in the case of those tissues such as nerve, in which the lactic acid formed in anaerobiosis cannot be removed in oxygen (Gerard and Meyerhof, 1927). In tissues capable of oxidatively removing lactic acid we must assume that the concentration on the inside of the cell and therefore the degree of inhibition is determined by the setting up of a dynamic equilibrium between the diffusion of HCN (or NaCN) into the cell, and its expulsion from the cell. The latter factor is determined by the rate of formation of lactic acid, the rate of its diffusion out of the cell and the rate of its oxidative removal (involving in turn the rate of inward diffusion of O_2). It is possible that herein is to be found the explanation of Dixon and Elliott's curves which show a constant though only partial inhibition by cyanide. As soon as the local concentration of lactic acid inside a cell has increased to a certain point, most of the HCN will have been expelled, allowing that locus to escape momentarily from inhibition (since cyanide inhibition is readily reversible), which in turn permits oxidative removal of the lactic acid. This, together with outward diffusion of lactic acid sets the stage for the re-entry of HCN and the genesis of another cycle of events. Upon such an explanation, varying the

cyanide concentration would have comparatively little effect except in the case of tissues incapable of oxidative removal of the lactic acid.[2]

2. THE EFFECT OF CYANIDE UPON THE PROPAGATION OF THE ACTION POTENTIAL.[3] Since cyanide is capable of inhibiting nerve respiration practically completely one would expect that the ability of cyanided nerves to conduct the impulse would be affected in the same way as in asphyxiation, i.e., by complete failure after a variable period. This has been found to be the case.

Sciatic nerves of large bull frogs, R. clamitans and R. catesbiana were placed in the hard rubber chamber previously described (Schmitt, 1930) in such a way that the nerves dipped under the surface of solution between the stimulating and lead-off electrodes. The action potential wave was recorded by means of the cathode ray oscillograph. Due to the long conduction distance (3–6 cm.), considerable separation of waves occurred. Although photographs were made of the compound action potential waves in some experiments, the procedure usually followed was that of measuring the height of the spike of the action potential. This, of course, gives no information as to the height of the action potential in the treated region. In a subsequent paper a detailed report will be made of the effect of cyanide upon the shape of the compound action potential wave, for some indication has been found that cyanide affects the larger fibers differently than the smaller fibers.

Neutral NaCN solutions were added to Ringer solution buffered with bicarbonate so that the final pH was 7.8. Every 20 minutes the cyanide solution was withdrawn from the chamber and replaced by fresh solution to prevent change of concentration by escape of cyanide.

As in the case of asphyxiation, nerve shows a preliminary rise in action potential in cyanide, which is followed by a fall to extinction. This is especially true of the more dilute solutions; concentrations as high as N/100 seldom show the preliminary rise. From the curves in figure 7 it appears that there is little difference in the time to extinction whether the concentration is N/100 or N/2000. When the solution is more dilute than N/5000, however, the time to extinction is greatly prolonged and in most cases extinction was never realized. Since the solutions were frequently changed, the concentration of cyanide in the bath was constant. Hence it is unlikely that the shape of the curves is conditioned entirely by the factor of diffusion across the perineural membrane. In the first place the

[2] Since sending this paper to press a paper by Warburg has appeared (Biochem. Zeitschr., ccxxxi, 493, 1931) in which further reasons are adduced to show that incomplete cyanide inhibition cannot be regarded as proof that respiration is not mediated entirely by heavy metal catalytic systems.

[3] This portion of the work was carried on at the Marine Biological Laboratory, Woods Hole, Mass., during the summer of 1930.

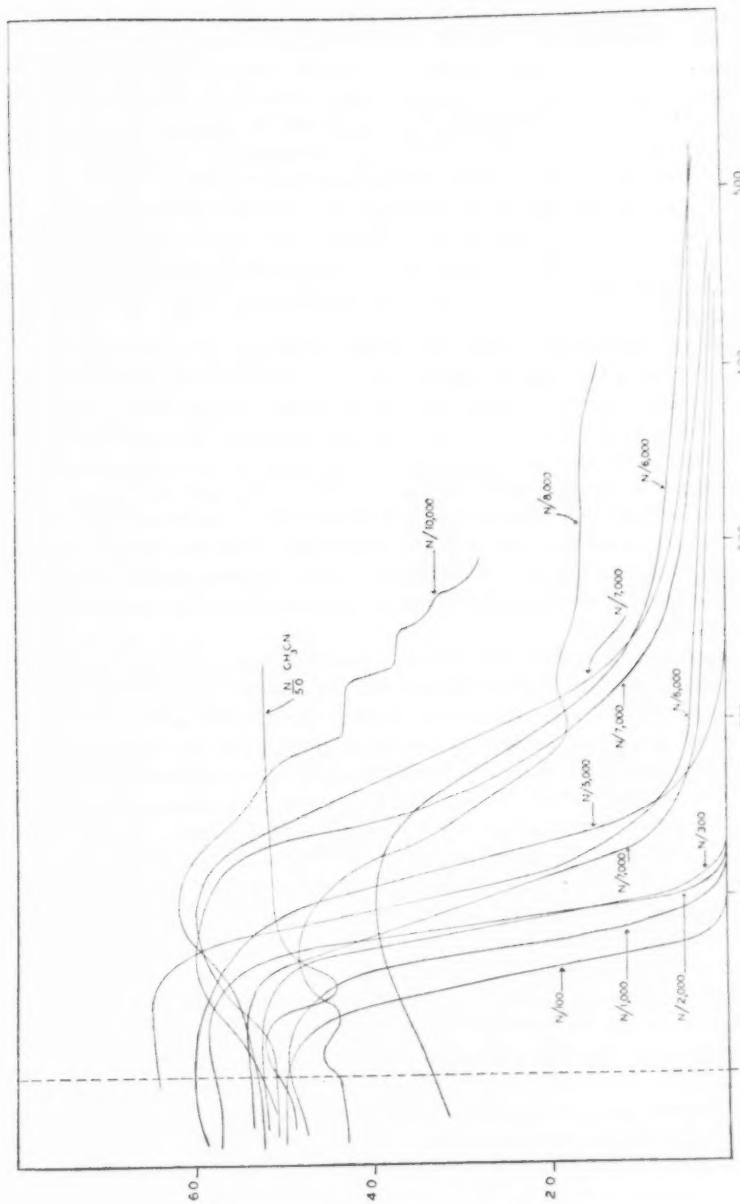

Fig. 7. Effect of cyanide upon action potential of bull frog nerves. Ordinates, deflection of action potential in millimeters on Braun tube; abscissae, time in minutes. Cyanide Ringer solution was substituted for Ringer solution at time, zero. Readings were made at least every ten minutes.

curves of failure in pure N_2 and in N/100 NaCN are practically superimposable. Furthermore, since the cyanide concentration in the bath is maintained constant, failure should occur in the higher dilutions within limits fairly predictable by the diffusion equation (see Hill, 1930), whereas the curves show that it is practically impossible to abolish the action potential in these nerves with solutions less concentrated than N/8000. It seems more likely that when the concentration becomes less than N/5000, lactic acid production in the inner fibers suffices to drive out the HCN and thus to prevent inhibition. The step-wise curve obtained with the N/10,000 NaCN probably indicates the simultaneous failure of groups of fibers as suggested for similar phenomena found in asphyxiated nerves by Gerard (1930).

The cyanide failure of nerves was seldom reversible by replacing the cyanide solution in the bath by Ringer solution. In most cases, however, if the nerve were removed from the apparatus entirely and placed for a time in Ringer solution at least partial recovery was obtained. It appears that the cyanide diffuses into the nerve beyond the point of immersion in the bath and that this cyanide must be removed before recovery will occur.

Since acetonitrile had no effect on nerve respiration it would be expected that it would have no effect on the action potential. This was found to be the case as is shown in figure 7. Evidently only isonitriles are effective in muzzling the heavy metal catalytic system responsible for the production of the action potential.

3. THE EFFECT OF METHYLENE BLUE. Following the work of Harrop and Barron (1928), Warburg (1930) showed that in the case of red blood corpuscles, methylene blue may take the place of the autoxidizable catalyst. Under these circumstances the O_2 uptake may be little decreased even under the influence of cyanide. In the presence of a sufficiently large amount of hemoglobin-like, nonautoxidizable substance capable of being oxidized by the methylene blue, the oxidation of sugar added to the cells was little interfered with by cyanide.

Methylene blue in 0.002 per cent solution may increase the oxygen consumption of resting nerves, and in several experiments the oxygen uptake of stimulated nerves was markedly increased, although this was the exception rather than the rule (Schmitt, 1929). Gerard (1930) states that the oxygen consumption may be increased as much as 70 per cent by methylene blue and that the addition of sodium lactate may cause still further increases. Stimulation of stained nerves had no effect on the oxygen uptake. Although Gerard (1930) does not state the concentration of the methylene blue nor give any other data, he mentions that this dye is capable of diminishing the inhibitory effect of cyanide on the oxygen consumption of resting nerves. In another place, using methylene blue not with the idea of evoking its *catalytic* action but simply as a hydrogen

acceptor, Feng and Gerard (1930) in a single sentence state that methylene blue is incapable of preventing the abolition of the action potential in cyanided nerves.

Considering these statements in the light of the recent paper of Warburg, Kubowitz and Christian (1930) it would appear that whereas the resting metabolism of nerve may be mediated in part at least by a nonautoxidizable (hematin ?) component which methylene blue can oxidize, the action potential mechanism involves no such component, or is not amenable to experimentation in this manner. Preliminary investigations have confirmed the statement that cyanided nerves stained with methylene blue fail just as rapidly as unstained cyanided nerves (see fig. 6). In further investigations on the catalytic effect of methylene blue, however, the oxygen uptake and the action potential are being recorded simultaneously in an effort to test the thesis that the action potential results from reactions distinct from those which cause resting oxygen uptake in nerve and take place, perhaps, at specific interfaces.

SUMMARY

1. The oxygen consumption of resting nerves of the green frog may be practically completely inhibited by cyanides. This is true whether the nerve is suspended in Ringer solution or in phosphate or bicarbonate buffer, and whether the measurements are made under physiological CO_2 tension or at zero CO_2 tension.

2. The action potential is abolished in N/100 NaCN about as fast as in pure nitrogen, e.g., in about 100 minutes. N/5000 NaCN is still capable of abolishing the action potential, although more dilute solutions act much more slowly and never completely block it.

3. Cyanided nerves stained in methylene blue fail just as rapidly as do unstained cyanided nerves. The possible catalytic action of the dye is discussed.

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BASAL METABOLISM AFTER THYROXIN IN SYMPATHECTOMIZED ANIMALS

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The increased metabolism following thyroxin injections may be explained as due to the action of the drug either directly upon the tissues or upon some intermediate organ which itself stimulates cellular oxidations. Aub, Bright and Uridil (1922), studying the problem, decided that the high metabolism produced by thyroxin could not be explained by muscular activity, muscular fibrillation or increased muscle tonus and that the adrenal glands were not essential to the maintenance of the high metabolic rate induced by thyroxin. They believed that the drug was a general metabolic stimulant. This conclusion has been in part substantiated by later work on isolated tissue. Ahlgren (1926) noted that muscles of thyroidectomized animals showed an increased oxygen consumption after thyroxin, whereas muscles from normal animals did not show this increase. And Reinwein and Singer (1928) found that thyroxin produced a rise in metabolism of isolated liver cells.

Observations on hyperthyroid cases, on the other hand, often indicate excessive activity of the sympathetic nervous system, and this has led some workers to believe that thyroxin acts through the medium of this part of the automatic system. The causal relations are not clear, however; thus Kessel, Hyman and Lande (1923) state that "while clinical manifestations of disturbances of the involuntary nervous system are often associated with thyroid hyperplasia, there is no reason to believe that the thyroid enlargement is causative and many reasons for thinking that it is secondary and symptomatic." Wilson and Durante (1916) found degenerative changes in cells of the superior cervical ganglion in patients who died of exophthalmic goiter. Again the question rises as to the rôle of the sympathetic system in the complex. Abderhalden and Wertheimer (1927) believe that thyroxin excites the sympathetic nervous system and thus indirectly raises metabolism. Their conclusion is based on experiments which showed no metabolic increase after thyroxin when participation of the sympathetic system was excluded by ergotamin. The effects of ergotamin, however, may not be limited to exclusion of the sympathetic nervous system (Moore

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and Cannon, 1930). A more satisfactory method of examining the question of sympathetic involvement in the action of thyroxin would be a study of the metabolic effects of thyroxin after complete sympathectomy.

In our animals the entire thoraco-lumbar chain on each side was removed, thus disconnecting the sympathetic fibers from the central nervous

TABLE 1

DATE	SYMPATHECTOMIZED			NORMAL		
	Weight	Calories	Calories per kgm.	Weight	Calories	Calories per kgm.
	kgm.	*per hr.*	*per hr.*	*kgm.*	*per hr.*	*per hr.*
	Cat 295			Cat 66		
May 19				2.8	5.95	2.12
May 21				2.8	5.93	2.12
May 23	4.2	8.33	1.98			
May 23	2.5 mgm. thyroxin, subcutaneously			2.5 mgm. thyroxin, subcutaneously		
May 26	2.0 mgm. thyroxin, subcutaneously			2.0 mgm. thyroxin, subcutaneously		
May 27	3.8	10.83	2.85	2.6	7.30	2.81
May 29	3.8	10.49	2.76	2.6	8.14	3.13
	Cat 107			Cat 100		
May 7	3.1	6.33	2.04			
May 9				2.4	5.06	2.11
May 19	3.1	6.63	2.14	2.4	5.39	2.25
May 20	2.5 mgm. thyroxin, subcutaneously			2.0 mgm. thyroxin, subcutaneously		
May 21	2.5 mgm. thyroxin, subcutaneously			2.0 mgm. thyroxin, subcutaneously		
May 23	3.0	9.79	3.26	1.9	6.62	3.48
May 23	2.5 mgm. thyroxin, subcutaneously					
May 27	2.0 mgm. thyroxin, subcutaneously					
May 29	2.6	8.27	3.18			
June 5	2.2	5.48	2.49			

system and at the same time removing the cell bodies from most of the sympathetic fibers. The outlying abdominal and cervical ganglia were left, which meant that most of the sympathetic nerves to the abdominal viscera and to the head and neck did not degenerate, though they were without central connection. It left the possibility that thyroxin might act upon these remnants and thus raise metabolism. But the oxygen con-

sumption of the alimentary canal, pancreas, spleen and liver amounts to 2.74 cc. per minute for the average fasting cat (calculated from data given by Barcroft and Shore, 1912) or one-eighth of the total basal metabolism of a cat weighing three kilograms. Since a large part of the remaining sympathetic nerve endings lie in this region, it is not to be expected that thyroxin could act through these and produce a 50 per cent increase in the total basal metabolism.

TABLE 2

DATE	SYMPATHECTOMIZED			NORMAL		
	Weight	Calories	Calories per kgm.	Weight	Calories	Calories per kgm.
	kgm.	*per hr.*	*per hr.*	*kgm.*	*per hr.*	*per hr.*
	Cat 7			Cat 66		
October 29	2.1	4.59	2.19	2.9	5.71	1.97
October 30	2.0 mgm. thyroxin, subcutaneously			2.0 mgm. thyroxin, subcutaneously		
October 30	2.1	4.65	2.21	2.9	6.81	2.35
October 31	2.2	5.40	2.45	3.0	6.84	2.28
November 1	2.5	5.04	2.02	3.0	6.06	2.02
November 3	2.7	4.93	1.83	3.3	5.47	1.66
November 3	2.0 mgm. thyroxin, subcutaneously					
November 4	2.1	6.82	3.25			
November 5	2.1	5.82	2.77	2.9	5.17	1.78
November 6				?	5.34	1.84
	Cat 66			Cat 95		
December 16	2.3	4.05	1.76	3.1	6.18	1.99
December 18	2.4	4.03	1.68	3.05	6.06	1.99
December 18	2.1 mgm. thyroxin, subcutaneously			2.9 mgm. thyroxin, subcutaneously		
December 19	2.4	6.21	2.59	2.9	6.89	2.38
December 23	2.2	5.10	2.32	2.7	5.98	2.21
January 6	2.3	4.31	1.87	2.9	5.20	1.79

Note that cat 66 was first used as a control animal and then sympathectomized, and that the results are not far different in the two cases.

RESULTS. The metabolism determinations reported were made in a closed-circuit apparatus, oxygen consumptions alone being used. A few open-circuit determinations have indicated that this method is entirely satisfactory with fasting animals.

At first we tried to produce a marked increase in the metabolism of our animals by repeated injections of thyroxin. Table 1 shows these results.

The response to thyroxin is the same in sympathectomized and normal animals. Single injections give similar results, though less striking (see table 2).

CONCLUSION

Removal of the thoraco-lumbar sympathetic chains does not prevent the usual rise in metabolism after thyroxin.

This work was done at the suggestion of Dr. W. B. Cannon.

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A BELATED EFFECT OF SYMPATHECTOMY ON LACTATION

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In an article published in 1929, Cannon, Newton, Bright, Menkin and Moore reported that after the removal of the sympathetic nervous system of the cat, the dog and the monkey the behavior and abilities of these animals are very little changed in many important aspects. They eat well, their digestive processes continue normally, their basal metabolism remains at approximately the usual level, the percentage of blood sugar is not altered—indeed, they live in the conditions of the laboratory, indefinitely, quite as normal animals.

In this paper it was also reported that sympathectomy does not prevent the female cat from carrying on her functions of reproduction and lactation. The observation on which this statement was based was made on a cat from which the last portion of the sympathetic was removed only about six weeks before the birth of her two kittens. She did, indeed, nourish one of her offspring until it was capable of foraging for itself. The other kitten was accidentally killed, but the assumption seems reasonable that if it had continued to live, the mother would have continued to nourish it without difficulty. Some further observations have led to new facts which modify our attitude regarding the effect of sympathectomy on lactation, and we wish to record them and qualify the statement made in the publication cited above.

The first observation which was inconsistent with our published statements was made on a bitch which had been deprived of her sympathetic system in three stages,—November 9, 1928; December 7, 1928 and January 8, 1929. On July 11, 1929, she gave birth to nine pups. Soon after their birth the young began to die, and in a few days only three were left. We found that they were not gaining in weight, and on examining the mother learned that the milk in her breasts was slight in amount, and a thick, viscid fluid, with the consistency of thick cream. We tried to keep the pups alive by artificial feeding, but only one survived. He was very vigorous, and attacked his mother's breasts energetically,—pushing, pulling and sucking one after another in his efforts to obtain nourishment. We followed his weight, which is recorded in figure 1, curve *A*, and found that

although he was the only pup she had to feed, his mother's milk was sufficient to permit him to gain only slowly. As soon as he was able to drink milk, however, he commenced to gain more rapidly. There are few obtainable data about the growth of puppies, though it is generally understood that those borne by a bitch kept under kennel conditions are smaller than those from mothers having free range in the open. According to a table

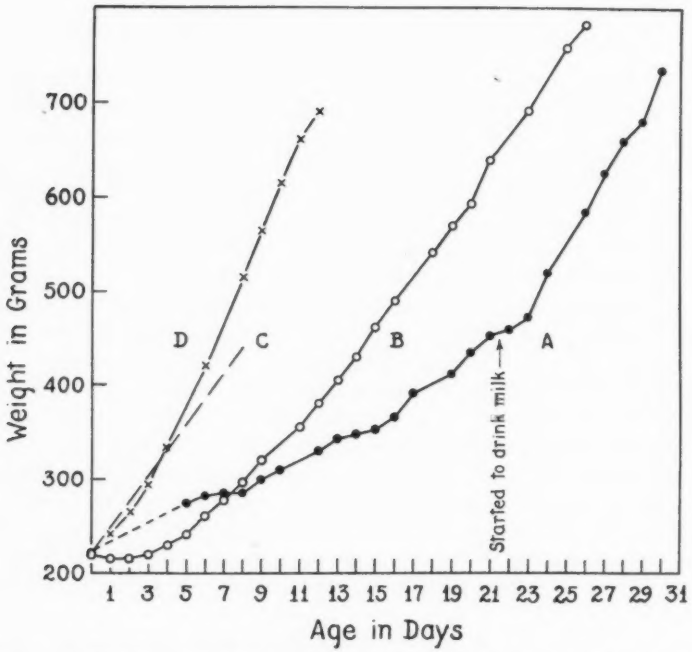

Fig. 1. *A*. Weight curve of the only surviving pup of a litter of nine, born of a sympathectomized bitch, who had been in the laboratory over a year. *B*. Weight curve of the only surviving pup of a litter of five, born of a normal bitch, who also had been in the laboratory over a year. *C*. Weight curve suggested for normal pups. *D*. Average of weights of two pups of a normal bitch, who had been in laboratory for only a few months.

compiled by Bunge (see Lusk, 1928), a new-born dog should double its weight in eight days (fig. 1, curve *C*), but we know nothing about the breed of either his dogs or ours. We have for comparison, however, the puppy of a normal dog of 12 kilos. This dog had been living in the laboratory about the same length of time as the sympathectomized dog, whose weight was 15 kilos. The puppy was about the same weight as the one whose

mother had been sympathectomized, but, after a three-day delay, it gained steadily on its mother's milk, and at a much faster rate than the other, even though its mother was the smaller of the two dogs (see fig. 1, curve *B*). The weight of two other puppies is averaged in curve *D*. Their mother was a small normal dog of 10 kilos who had been in the laboratory only a few months. These puppies gained steadily on their mother's milk, much more than doubling their weight in eight days.

On February 11, 1930, about twelve months after sympathectomy was complete, the sympathectomized dog gave birth to nine more pups, none of which equaled her earlier surviving pup in birth weight, the average being 160 grams, as compared with about 220 grams. These all died within two days. Examination of the mother's breasts gave no evidence of any milk being secreted. Her maternal instincts seemed fully developed, however, for she was troubled and watchful when the pups were taken away, and was contented again when they were returned.

Another case was that of a cat (107) from which the last of the sympathetic had been removed twenty months before she gave birth to three kittens. In this animal also the mammary glands were not prepared to give milk. The areolae remained flat and dry, and the nipples small and slender. Associated with this condition was a remarkable indifference of the mother to her young, with regard to the function of lactation. If she was made to lie down and the young were brought to her to suckle, she would rise as soon as constraint was removed and leave the kittens without showing the least interest in their welfare. To their cries of hunger she paid no attention.

From the foregoing observations it would appear that sympathectomy does not interfere with the processes of reproduction in the female, but that it may disturb lactation, and that in the cat and dog the disturbances are more profound the longer the time after the sympathetic system is extirpated.

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THE EFFECT OF DIFFERENT PER CENTS OF PROTEIN IN THE DIET

IV. Reproduction[1]

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Five groups of rats, designated I, II, III, IV and V, were fed continuously throughout their life span and several succeeding generations on carefully prepared synthetic diets containing 10, 14, 18, 22 and 26 per cent of protein respectively. All the known amino acids and vitamins were contained and each diet had an energy value of 3.82 calories per gram. A detailed description of the plan and procedure of the experiment was given in the first paper of this series (Slonaker, 1931).

In this paper, which deals with the effect on reproduction, we will give the results of the 18 original pairs in each group and of such rematings as were necessitated on account of the death of original animals.

Mortality. Table 1 gives the total number of animals of each sex which were required in each group to carry the experiment through the average life span. It is noticed that with the exception of group IV the mortality of the males was greater than that of the females. It is also seen that the males of group III and the females of groups II and III required the fewest replacements of any of the other groups. The mortality in each sex was greatest in group V and was only a little less in group I. This suggests that the average life span for each group would correspond closely to the number of replacements required. This in general was found to be true, but a complete discussion will be given in a later paper dealing with the life span and cause of death.

Sterility. It was soon noted that some of the pairs failed to produce litters. In order to determine which sex was inefficient or if both were at fault, the male was tested with a fertile female and the female of the pair

[1] This research has been assisted by the Department of Physiology, the National Research Council through the Committee for Research in Problems of Sex, The National Live Stock and Meat Board, the Fleischmann Company and their successors, The Standard Brands, Inc., and the coöperation of Albers Bros. Milling Company.

with a productive male. The per cent of fertile males and females was thus determined. The per cents for each sex in all the matings of each group are given in table 1. It is noticed that the females in each group had a higher per cent of fertility than the males of the same group. It is also seen that the order of efficiency of the diets was the same in each sex. From most efficient to least the order of the value of the diets was II, I, III, IV and V. Besides these animals which were sterile at all ages there were others which became sterile after the birth of a single litter. This is indicated in table 2 by the average number of litters thrown by the productive females. These data indicate that the diet containing 14 per cent protein was the optimum and that reproduction was more and more seriously interfered with as the protein content increased.

TABLE 1

Giving the average ages in days of fertile animals at first and last litters, the length of the reproductive span, the total number of matings and the per cent of fertile animals in each group of the first matings

GROUP	FERTILE ANIMALS								TOTAL MATINGS			
	Number		Average age at first litter		Average age at last litter		Average reproductive span		Number		Per cent fertile	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
I	21	23	144	150	462	435	318	285	25	24	84	96
II	20	21	136	143	490	494	354	351	23	21	87	100
III	14	17	138	275	431	461	293	186	22	21	64	81
IV	10	14	318	345	442	506	124	151	23	23	43	61
V	9	13	381	372	450	499	69	127	27	26	33	50

Reproductive span. The data given here have reference only to fertile animals. Obviously sterile animals would have no reproductive span. Table 1 gives the number of fertile animals of each sex in each group which will now be considered. We have used the time elapsing between the ages at birth of the first and last litters as representing the reproductive span. Strictly speaking this time should be increased approximately 22 days (the average gestation period). Table 1 shows that with the exception of group V the male became sexually mature at an earlier age than the female of the same group. It is also seen that both sexes in group II were fertile at an earlier age than any of the other groups. Also that diets containing either less or more than 14 per cent of protein retard the age of sexual maturity as indicated by the age at which the first litter was born. This retardation in group V was extended approximately 2.7 times that of group II. The range in age at first litter showed great variation. The ranges in the males in groups I to V were: 129–328; 145–371; 152–325;

135–395; 190–582 respectively. For the females they were 87–274; 93–262; 157–733; 188–489; 199–535 respectively. The ages at birth of the last litter also show a wide divergence. For the five groups in order the males were: 254–676; 266–678; 181–659; 192–788; 190–625; and the females were: 178–754; 265–794; 171–733; 340–632; 304–714 respectively. The age at last litter may be considered the age at which the animal became sterile. These figures show a female in group I mating at 65 days and one in group II at 71 days of age. They also show that the oldest age at which a litter was born is much greater than that generally given for the rat. The age of 600 days is usually considered about the limit of fertility. However, the number of males which exceeded 600 days at the last litter in the different groups from I to V were 3, 5, 2, 1, and 1; and the females were 4, 6, 3, 4 and 3 respectively. These numbers represent the following per cents of fertile animals: males, 14, 25, 14, 10, and 11; females, 17, 28, 18, 27, and 23 per cent respectively. This

TABLE 2

Giving the number of fertile females, the total young and number of litters, and the average number and size of litters in each group

GROUP	FERTILE FEMALES	TOTAL YOUNG	LITTERS		
			Total number	Average number	Average size
I	23	642	122	5.30	5.26
II	21	623	121	5.76	5.15
III	17	314	67	3.94	4.68
IV	14	184	42	3.00	4.38
V	13	148	38	2.92	3.90

shows that group II had the greatest per cent of both males and females which were fertile beyond the age of 600 days than any of the other groups. On examining the average length of the reproductive span as given in table 1 we note that from longest average span to the shortest the order in both sexes was group II, I, III, IV and V. These data indicate that a diet containing slightly less than 14 per cent protein would be the optimum for producing the longest reproductive span in both sexes of the rat. It is also noted that with the exception of groups IV and V the reproductive span was longer in the males than in the females of the group.

Reproduction. The discussion which follows refers only to the fertile animals which produced litters. In table 2 we have given the total number of young born, the total number of litters and the average number and average size of the litters in each of the groups. This shows a rapid decrease in the number of fertile females which threw young from group II to group V. A corresponding decrease in the total young born would follow

unless compensated for by an increased productivity of the females on the richer diets. That such a compensation did not occur is proved by the average number of litters and the average size of the litters in the different groups. There was quite a difference in the range of the number of litters in the groups. In order from group I to V the range from fewest to greatest number of litters delivered by a single female was 2–10, 2–9, 1–9, 1–6, and 1–7 respectively. In group IV there was only one which had a litter of six and three had a litter of one. In group V there was but one female which had a litter of seven and three with litters of one each. These figures show that in groups IV and V not only was the average size of the litter less but also the maximum litter born was less than in any of the other groups. This indicates that as the per cent of protein in the diet increased above 14 per cent the effect on the number and the size of the litters became more and more inhibitory.

TABLE 3

Giving the number of litters, males and females, the average number for each mating and the sex ratio of the young born in each group

GROUP	NUMBER OF LITTERS	YOUNG BORN			AVERAGE YOUNG PER MATING		MALES TO 100 FEMALES
		Males	Females	Total	Total matings	Fertile matings	
I	122	324	328	652	27.2	28.3	99:100
II	121	312	311	623	29.6	29.6	100:100
III	67	141	173	314	14.9	18.5	81:100
IV	42	88	96	184	8.0	13.1	92:100
V	38	66	86	152	5.8	11.7	77:100

In table 3 we have given the total number of males and females of generation 1 born, the average number of young per mating and the sex ratio in each group. This table shows that group II had the greatest average number of young per pair in both total and fertile matings. It was followed closely by group I. As the per cents of protein increased above 14 per cent there was a marked progressive reduction in the average number of young born.

The normal sex ratio in the albino rat is approximately 106 males to 100 females (King, 1911; Cuénet, 1899). Table 3 shows that none of our groups attained this ratio. The nearest approach was in group II followed closely by group I. The young of groups III, IV and V had a sex ratio considerably below normal and corresponded closely to the ratio we found with parents on a restricted diet (Slonaker and Card, 1923). The sex ratio of all groups indicates that there was a greater pre-natal mortality of the males than of the females. The increased inhibition of reproductive ac-

tivities caused by the higher per cents of protein in the diet suggest either a reduction in the number of ova liberated at oestrus, or a lowering of the vitality of the sperm, the ova, or both to such an extent that fertilization only occasionally results. Further investigation will be necessary to determine this.

In table 4 are given the number and per cent of males and females weaned and the sex ratio of weaned animals in each group. With the exception of group V the per cent of males weaned exceeded that of the females of the same group. This indicates that after birth the vitality of the males was greater than that of the females. A comparison of the sex ratios at birth and at the weaning age in general substantiates this. It is again noted that group II was able to rear to the weaning age a larger per cent of the young born than any of the other groups. This may have been due to greater vitality of the young, but more likely to the mother's greater ability to nurse the young.

TABLE 4

Giving the number of litters, males, females, per cents, average ages, and sex ratio of the young weaned in each group

GROUP	LITTERS		YOUNG WEANED			PER CENT WEANED			AVERAGE AGE	MALES TO 100 FEMALES
	Born	Weaned	Males	Females	Total	Males	Females	Total		
I	122	67	197	191	388	62	59	60	26	103:100
II	121	81	232	214	446	74	69	72	25	108:100
III	67	34	76	84	160	54	49	51	24	90:100
IV	42	23	56	55	111	64	57	60	26	102:100
V	38	16	32	43	75	48	50	49	28	75:100

Gestation period. Owing to the fact that the pairs in stationary cages were constantly together and the time of coitus could not be ascertained without constant observation we were unable to determine the length of gestation in these animals. We were, however, able to note the exact hour of coitus in the ten females in each group which were isolated in revolving cages and which were removed for mating. Since the young were born at all hours of the day and night it was only by chance that we were able to observe the exact hour of birth and determine the true gestation period. The young begin nursing within an hour or so after birth. If they have nursed the presence of milk is easily recognized. We were therefore able to estimate rather closely the hour of birth of the litters which were not observed at birth and to determine their approximate gestation periods. In table 5 we have given the results of the litters with which we were able to deal.

We have previously shown (Slonaker, 1925) that the size of the litter

modifies the length of the gestation period. We found that in small litters, ranging from 1 to 6 and averaging 4.25 each, the length of gestation averaged 21 days 23.8 hours. In large litters, ranging from 7 to 14 and averaging 9.12 each, the length of the gestation period averaged 21 days 11.38 hours. We also stated that in general young mothers had a shorter gestation period than older females when the size of the litters was approximately the same. By consulting table 5 it is seen that the average age at which the litters were born in the different groups increased from group I to group V. This would tend to increase the length of the gestation period in the same order. In table 2 we showed that the average size of the litters decreased from group I to group V. The differences in the ages and in the size of the litters may have been sufficient to cause the lengthening of the gestation periods as given. There was one female in group III which had an abnormal delivery lasting two days. This accounts for the long gestation period of 25 days and brought the average for this group longer than it normally would have been. These results seem to indicate that the diets, per se, had little or no effect on the length of the gestation period, but did exert an influence by modifying the size of the litters and the age of the mothers at the time of delivery.

TABLE 5

The number of litters, the average gestation period, the shortest and the longest gestation periods in each of the groups

GROUP	AVERAGE AGE	NUMBER OF LITTERS	LENGTH OF GESTATION							
			Average		Shortest		Longest		Range	
			Days	Hours	Days	Hours	Days	Hours	Days	Hours
I	311	51	22	10	20	00	24	00	4	00
II	359	61	22	10	20	20	24	00	3	4
III	404	55	22	18	21	20	25	00	3	4
IV	478	29	22	16	21	20	23	15	1	19
V	488	23	22	14	21	16	24	00	2	8

SUMMARY

When five groups of rats, I, II, III, IV, and V, were fed on diets containing 10, 14, 18, 22 and 26 per cent of protein respectively the following results were obtained.

1. The mortality of the males of the first matings was in general greater than that of the females. The mortality of both sexes was least in groups II and III. It was greatest in group V.

2. Sterility was more pronounced in the males than in the females in each group. Group II had the highest per cent of fertile animals in each

sex and group V the lowest per cent. The order of fertility in both sexes from highest to lowest per cents was group II, I, III, IV and V. The extreme per cents of fertility in the males were group II, 87 and in group V, 33. In the females they were 100 and 50 per cent respectively.

3. From longest to shortest reproductive spans the order in both sexes was group II, I, III, IV and V. The males of groups I, II and III had longer reproductive spans than the females of these groups. In groups IV and V the reverse obtained.

4. The average number of litters and the average number of young born per pair from greatest to least was in the order of groups II, I, III, IV and V. The size of the litter was in the order of I, II, III, IV and V.

5. The sex ratio of the young at birth was highest in group II and lowest in group V. The ratio was below normal in all groups.

6. Results suggest a greater pre-natal mortality of males than of females.

7. With exception of group V a higher per cent of males in each group were reared and weaned than females. Group II had the greatest per cent of young weaned (72 per cent) and group V the lowest (49 per cent).

8. Post-natal mortality was in general greater in the females than in the males indicating a greater vitality of the males.

9. The length of the gestation period was the shortest in groups I and II and increased in the order of V, IV and III. The differences in the length may be due to the age of the mother and the size of the litter.

10. In general the order of efficiency in reproduction of these five diets from greatest to least was II, I, III, IV and V.

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THE INSPIRATORY AUGMENTATION OF PROPRIOCEPTIVE REFLEXES

A Study of the Knee Jerk and the Achilles Reflex

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It has long been recognized that reflex responses to successive stimuli of constant intensity exhibit great variability. Impulses originating in the skin, muscles, tendons, viscera, and in various parts of the central nervous system itself, are more or less constantly impinging on the central portions of the spinal reflex arcs, and modifying their activities, either in the direction of augmentation or of inhibition. The respiratory center constantly discharges, rhythmically, a succession of impulses which excite to activity a large group of muscles. That these impulses irradiate to other parts of the central nervous system, and through them modify the activities of skeletal muscles not concerned in respiration, has recently been demonstrated. We presented to the XIIIth International Congress of Physiology (1), a report in which it was shown that the knee jerk, the Achilles reflex, the scratch reflex, parathyroid tetany, and shivering frequently show a definite augmentation during inspiration. While this work was in progress, Strughold (2), whose observations were then unknown to us, reported similar findings with reference to the knee jerk in man. The possibility of a respiratory variation occurring in the knee jerk suggested itself to Lombard (3), but he failed to demonstrate it. His method of procedure was not so well adapted to this end, and furthermore, he may not have had suitable subjects. Richet (4) reported an inspiratory shiver rhythm, and Bazett (5) made an incidental reference to a respiratory rhythm in convulsions occurring in chronic decerebrate cats. Aside from these notations, the literature, so far as we have been able to learn, is silent with reference to the effects of inspiratory impulses on the activities of skeletal muscles other than those directly involved in breathing.

It is our purpose in this paper to report experiments and observations correlating variations in several proprioceptive reflexes with the phases of the respiratory cycle.

Material and methods. The observations were concerned chiefly

with the knee jerk, although a few short series were made on the Achilles reflex. These were selected because they lend themselves quite readily to quantitative measurement. The fact that both of the reflexes are obtainable in the spinal animal is proof that the basic central mechanism is spinal, and because of their brief latency, are short circuit reflexes. A comparative study of them in the normal human subject, and in the anesthetized, the decerebrate, and the spinal animal demonstrates clearly that they are profoundly affected by impulses originating above the cord levels. Our attention was concentrated on an analysis of the effect of impulses from the respiratory center.

The human subject, the dog, and the cat were used in our experiments. Nothing new in principle, in methods of stimulation, or of recording was developed. The solenoid hammer similar to the one described by Johnson (17), the gravity hammer, and the spring hammer were used for stimulation. The choice in any particular experiment depended upon the conditions and the main point of interest. The solenoid proved troublesome when electromyograms were being made. When the experiments of others were being repeated the attempt was made to duplicate their apparatus and procedure as accurately as possible. The same determinants served as guides in the choice of a method of recording, a variety, differing somewhat in principle, being employed. Extension of the leg was recorded by a lever and pulley system. The thickening of the quadriceps group was recorded by means of a rubber cuff and tambour, by a lever and pulley arrangement, and by direct shadow projection of the muscles. The isometric registration was accomplished through the use of a lever modeled after the one described by Fulton (6); however, instead of projecting the shadow of a fine needle, a small mirror was fastened to the lever, and from it a vertical beam of light was reflected to the slit of the camera. The electrograms were obtained in the usual manner with a string galvanometer. The use of shadow projection deserves comment since by this method the factors of inertia and inherent vibration which enter into any mechanical device for recording, are eliminated. The subject was placed in the supine position, with the long axis of the thigh almost vertical. The anterior surface of the thigh was made to cut through a cone of light playing on the slit of the camera. With the thickening of the quadriceps group the cord of the darkened segment moves in the horizontal direction, and will photograph as a spike on the moving paper. The respiratory movements were recorded by a pneumograph and tambour, and also by a lever and pulley system.

The basic observation. During normal inspiration both the knee jerk and the Achilles reflex are augmented. Figures 1 and 2 are records demonstrating this phenomenon in each of these reflexes. This basic observation has been demonstrated in normal men, in barbitalized dogs and cats, and in decerebrate cats. Some human subjects and some animal preparations

occasionally fail to show this augmentation at one time, but exhibit it under other conditions. An analysis of our data leads to conclusions which warrant the assumption that one might encounter very few, if any, failures if all the conditions could be accurately controlled. It has been shown by a number of observers (Lombard, 3; Lee and Kleitman, 7; Tuttle, 8), that in man during sleep the knee jerk is greatly diminished and frequently entirely abolished. Recently, Jacobson (9) has demonstrated that even in the waking human subject, in the proper state of relaxation, it may be impossible to elicit this reflex. Presumably, in the human subject at least, a certain degree of reinforcement is necessary before the knee jerk can be elicited. Excitement, pain, and other disagreeable experiences may augment, diminish or abolish the knee jerk.

Fig. 1

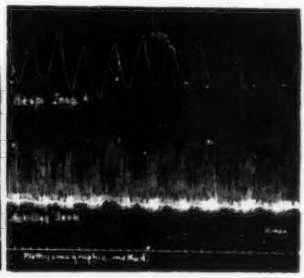

Fig. 2

Fig. 1. Inspiratory augmentation of the knee jerk during normal breathing. Barbitalized dog. Lower line time in seconds. x and y mark coördinate points.

Fig. 2. Inspiratory augmentation of the Achilles jerk. Human subject. Lower line time in seconds. x and x mark coördinate points.

Favorable and unfavorable conditions. The ideal conditions would be established if one could maintain a constant reinforcement sufficient to insure good response, thus giving a clear field for the manifestation of a rhythmical reinforcement such as appears during inspiration. The simultaneous play of a number of variables may either inhibit or mask a reinforcement which otherwise would appear rhythmically.

Our most consistent results were obtained with barbitalized animals. Some failures, however, were encountered even with this type of preparation. A study and analysis of our notes reveals rather clearly some of the factors responsible. The injury to the animal, and the posture in which it is placed and held incident to preparation, are the most important, being in some instances favorable, in others unfavorable. A number of times animals were brought into the laboratory, having received the dose of barbital some hours previously, limp, breathing quietly, and in good condi-

tion. A tap on the patellar tendon failed to elicit a reflex, or if so, only a very weak response. After the manipulations incident to the insertion of a tracheal cannula, tying the animal down, fixing the femur, and attaching the recording apparatus, sharp responses could be obtained. Other animals presenting the same condition when brought in, became restless and often rigid after preparation, and from them no rhythmical inspiratory augmentation could be obtained, only a very irregular, although sharp, response. Obviously the inspiratory effect was masked.

Bearing on the explanation of these variations, reference may be made to the studies on action currents in cut or injured afferent mammalian nerves recently reported by Adrian (10). He demonstrated that for some hours after injury, impulses, either regular or in groups pass along the nerve. Since in the process of insertion of a tracheal cannula and in fixing the femur many afferent nerves are cut and injured, the number and extent depending somewhat upon the operator's skill, a constant source of impulses is established. We have frequently observed an immediate and sustained acceleration of the heart after very slight operative procedures involving only skin incisions and very superficial dissection. It is therefore highly probable that the disturbances recorded by Adrian are at least in part responsible for the increased excitability of the central nervous system, and if they are constant and not too intense, may produce a favorable setting for the demonstration of rhythmical and periodic variations in reflexes. One is also reminded of the work of Pi-Suñer and Fulton (11), in which it was found that rotation of the head may profoundly affect the tonus of the quadriceps muscles, the nature of the effect depending upon the direction of rotation. In the restless animal, even though tied down, impulses from the neck muscles and from the bound limbs may either inhibit or augment a given reflex. These factors are not always subject to complete control, but with careful technique may be reduced to a constancy favorable to the demonstration of an inspiratory augmentation.

Inspiratory irradiation. We may now inquire whether the inspiratory augmentation of the knee jerk is due directly to the irradiation of impulses from the respiratory center, or is a reflex either from the respiratory tract itself or from the muscles involved in breathing, or whether it is due to mechanical changes in the muscles of the thigh incident to the respiratory shift in position or muscular tug.

Potentially, any afferent impulses reaching the central nervous system at the proper time may modify any reflex which happens to be elicited. It is therefore assumed that impulses from the respiratory tract and muscles, in addition to their effects upon the respiratory center itself, may modify the activities of other parts of the central nervous system, and thus be reflected in other bodily activities and reactions. Whether or not these impulses constitute the prime factors responsible for the inspiratory augmentation of the knee jerk is now our chief concern.

The vagi may be eliminated from consideration as prime factors in the inspiratory augmentation, for we have found that their section in the cervical region does not abolish the respiratory effects on the knee jerk, in fact the augmentation is often more pronounced after these nerves are severed. Figure 3 is a record from a barbitalized dog taken about two hours after double vagotomy. The augmentation is much more definite and marked than before the nerves were cut. Furthermore, stimulation of the central stumps of the vagi results in a sharp diminution in the knee jerk, coming on much too quickly to be accounted for by the blood changes due to the cessation of respiratory activity. These two facts, separately and together argue

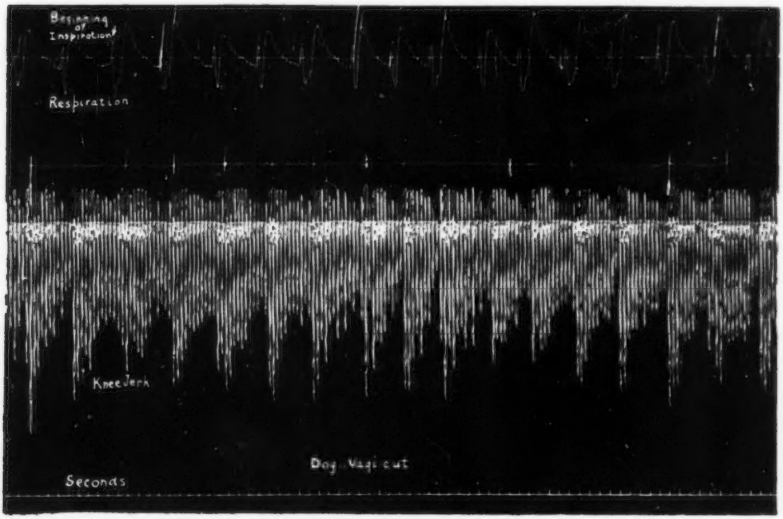

Fig. 3. Inspiratory augmentation of the knee jerk in a vagotomized dog

definitely against the augmentation being a reflex effect due to afferent pulmonary vagal impulses. They point, rather, to a possible inhibitory effect.

The evidence dealing with impulses from other parts of the respiratory tract and from the respiratory muscles is indirect. It is a well known fact that overventilation is followed by an apnea, the duration of which is dependent upon the acapnia produced. This is true, at least, for mild overventilation. The state of alkalosis which obtains at this time is favorable to spinal reflexes, there being no diminution, and frequently a definite sharpening. If during the apnea, artificial ventilation is induced, no inspiratory rhythm can be obtained in successive knee jerks, although the

moving mass of air, the changes in the bronchial tree and alveoli, and the stretch of the respiratory muscles are not unlike those obtaining during normal breathing. The conclusion is drawn that the respiratory center must be active in order to induce an inspiratory augmentation of the knee jerk.

It is also evident that changes in position and tension of the quadriceps muscles, brought about by the inspiratory shift in position and movements of the abdominal muscles, is not primarily responsible for the increase in the knee jerk. It is well known that a certain degree of tension of the muscles involved is necessary to establish a minimal threshold, and that the threshold varies with changes in resting tension. It is also common knowledge that the locus of concentration of the blow on the tendon is an important factor in determining the intensity of the response. Attempts were made with all our animals and human subjects to reduce variations in tension and position to a minimum. In most instances this was accomplished to the extent that no variations in the base line were recorded. By actual tests, such as manual manipulation of the leg and abdomen, it was found that much greater variations in position than those obtained spontaneously were necessary to produce a displacement or movement rhythm. The most conclusive evidence was obtained from our spinal animals. In none of these preparations did we obtain an inspiratory augmentation of the knee jerk or Achilles reflex, although the tension and postural changes were similar to those in the intact animals.

Another point, repeatedly observed and recorded, has a bearing on the question of reflex augmentation. In a number of records made early in the course of the experiment, the inspiratory augmentation appeared almost synchronously with the onset of the inspiratory movement. The inflation of the lungs and the stimulation of the muscle afferents had not yet taken place, which makes it obvious that the augmenting factor was directly associated with the discharge of impulses from the respiratory center.

Phase and duration of the augmentation. That the appearance of the increased reflex response in the early stage of an experiment is often practically synchronous with the onset of inspiration has already been mentioned. Many of our animal preparations were used for two days, and while a few showed sharp reflex excitability throughout this period, the condition of most of the animals declined gradually, and with this gradual development of poorer reactivity, the onset of the inspiratory augmentation appeared later and later in the phase of inspiration, and became progressively less demonstrable. It was not possible to elicit more than one or two responses during any single inspiration, but by a study of the responses through a large number of respiratory cycles, and making a composite picture, it became evident that the augmenting factor is in operation to the end of inspiration, and then declines rapidly.

Expiratory reinforcement and forced breathing. Concerning expiratory reinforcement of the knee jerk during normal breathing, we find that with the possible exception of one case, a human subject, we have encountered no clear evidence that it takes place. In this individual the effect was not pronounced, and since it came early in the expiratory phase, it is not entirely clear whether it was an expiratory effect or a late inspiratory one. We are inclined toward the latter view, for by forced breathing an inspiratory augmentation was brought out with little or no change from the normal during expiration.

On the basis that expiration is normally more passive than inspiration, one would expect it to have less effect on reflexes. At this juncture another consideration must not be overlooked. Bowditch and Warren (12) were able to demonstrate that if the patellar tendon is struck during a certain interval of time following the termination of the reinforcing act, the response is greatly diminished, the maximum inhibitory effect being attained from 0.6 to 0.9 second after the reinforcement ceases. This inhibitory state, however, does not completely disappear until several seconds have elapsed. It is probable, therefore, that following the inspiratory reinforcement there is established an inhibitory state falling within the expiratory phase which would tend to prevent, or at least diminish, any reinforcing effect.

In forced breathing, one might look for some reinforcement of the knee jerk during expiration, and by the same token, an increase in the inspiratory effect. In general these expectations have been realized, although we have never obtained an expiratory augmentation comparable in magnitude with that obtained during inspiration. Many unfavorable factors enter into a forced respiration experiment. When using the lower animals it is necessary to introduce resistances into the air channels to obtain the forced effect. Such procedures in a reflexly alert animal often produce effects which mask the reactions to be studied. It has been our experience that these procedures often result in an inhibition of the knee jerk, perhaps through the vagi. In the vagotomized animal this mechanism is eliminated, and it was in these preparations that we obtained the most consistent augmentatory results. The normal breathing after cutting the vagi is of the forced type, and in most of these animals we were able to record definite increases in the inspiratory augmentation, and a much less marked, although definite, expiratory augmentation.

With the human subject the proper conditions are also difficult to obtain. No resistance need be placed into the air channels, but considerable voluntary effort is required. This in itself, particularly in the untrained and inexperienced subject, produces changes which may even mask or abolish an inspiratory augmentation which was present during normal breathing. There is a marked tendency on the part of these subjects to overventilate,

which in itself tends to augment the knee jerk. Several subjects, after repeated trials, were able to carry out deep and forced breathing without developing any tenseness or alkalosis. In them, in the majority of tests, the inspiratory augmentation became more pronounced, or appeared, if absent during normal breathing. In no instance did we obtain an expiratory augmentation comparable to that obtained during inspiration. Figure 4 is a record from one of the experienced subjects. At the time this record was taken there was no evidence of a definite inspiratory augmentation during normal respiration, although at other times it was definitely demonstrable in this subject. It will be noted that with the induction of deep breathing a clear inspiratory augmentation appears, but the effect during expiration is questionable, there being only a slight increase during this phase of res-

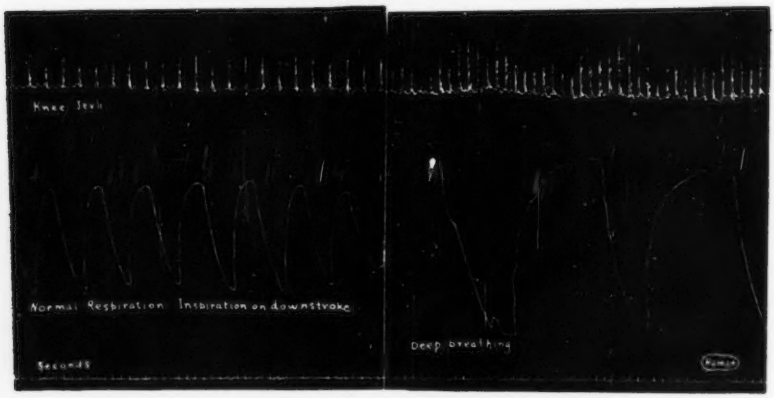

Fig. 4. Inspiratory agumentation of the knee jerk of the human subject, absent during quiet respiration, appears upon deep breathing.

piration, and were it not for its constancy throughout the whole length of the record, would not be seriously considered.

Relation between inspiratory augmentation and the strength of stimulus. On theoretical grounds, a maximal effect cannot be augmented. It is quite improbable that a maximal knee jerk, in the sense that all the moto-neurones and muscle fibers are involved, is obtained as a rule, even though further strengthening of the blow fails to elicit an increase in response. Many of the animals and human subjects studied, showing the inspiratory augmentation, were tested with stimuli ranging from below threshold to maximal or above. In several of our preparations we succeeded in so grading the strength of stimulus that reflex responses were obtained only during inspiration. Figure 5 is a record obtained in one of these experiments. The augmentation in practically all instances was most marked through

the range of stimuli causing weak and medium responses; it gradually diminished and occasionally was not demonstrable at all when the responses approached the maximum. Perhaps in these latter cases the true maximum was attained.

Rate of stimulation. Strughold, whose work was cited in the earlier paragraphs of this paper, studied the variations in the knee jerk in relation to the respiratory cycle from the standpoint of the rate of stimulation. From earlier work he had concluded that the total refractory period of the knee jerk is long,—from 3 to 6 seconds. He was able, using human subjects, to demonstrate an inspiratory augmentation of the knee jerk, only when the successive stimuli fell at intervals shorter than the total refractory period. He accounted for his results on the assumption that the refractory

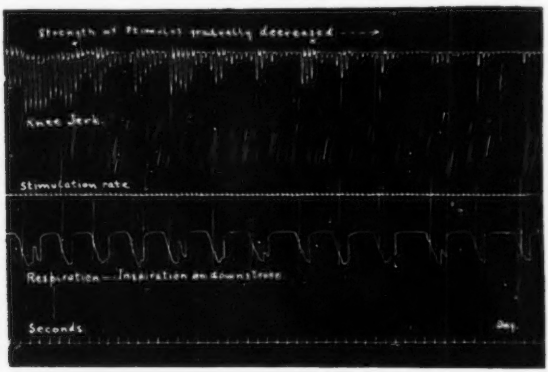

Fig. 5. The inspiratory augmentation of the knee jerk of the dog. By gradually weakening the stimulus the responses gradually diminish and practically cease during the expiratory phase but persist during inspiration.

period is shortened during inspiration. Before his work was known to us, in fact before it was published, we had adopted intervals of stimulation of from one to two seconds.[1] This choice was based upon our desire to obtain records which would contain enough responses during each respiratory cycle to show at a glance any correlation rather than to resort to the construction of composite graphs. We had, however, at various times obtained evidence which indicated that the inspiratory augmentation could be demonstrated when the successive stimuli fell at intervals much greater than the probable length of any refractory period. It therefore seemed

[1] A portion of the data used in this report was incorporated in a dissertation submitted to the faculty of Vanderbilt University by E. A. Blair in the spring of 1928 in candidacy for the degree of Master of Science.

desirable to investigate this question through a wide range of intervals of stimulation.

Two series of experiments were carried out. In one group human subjects and barbitalized dogs were used. The knee jerk was elicited and recorded in a variety of ways, including an almost exact duplication of those used by Strughold. In the second group, barbitalized dogs, both intact and spinal, were employed. The patellar tendon was freed from its tibial attachment, and fastened to an adjustable spring lever for recording purposes. The object of this procedure was to eliminate any mechanical effects of the contraction of the opposing flexors, a phenomenon occurring occasionally according to a number of observers. In the spinal animals

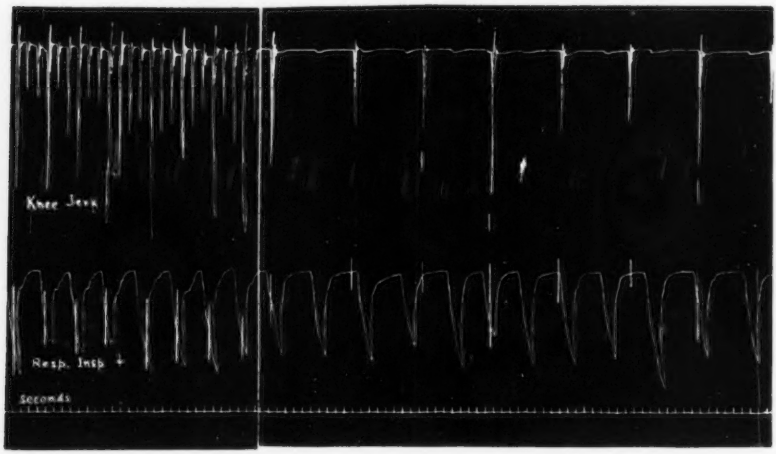

Fig. 6. The inspiratory augmentation of the knee jerk of the dog is demonstrable with rapid as well as with slow stimulation.

the cord was sectioned at about the last thoracic level, designed to leave the knee jerk arcs intact, but to cut them off from the reinforcing and inhibitory influences from higher levels as much as possible, and also to produce a minimum disturbance in the general circulation. Under these conditions variations in the refractory period should be considerably reduced. In both of these groups a range of rate of stimulation from one a second to one every twenty seconds, with gradations in strength from threshold to maximal, was used. A light solenoid hammer was constructed which enabled us to attain a speed of stimulation of four a second without any variation in strength. This was used on three animals in which the patellar tendon was cut loose from the tibia.

The results of these experiments showed that in all the animals and men

exhibiting the inspiratory augmentation, the phenomenon is clearly demonstrable throughout the whole range of stimulation, with no demonstrable difference in degree. We did not encounter the diminished mean height of response on rapid stimulation such as is shown in Strughold's report. Figure 6 is a record obtained from a barbitalized dog showing an inspiratory augmentation of the knee jerk. The briefest interval between stimuli is about one and one-fourth seconds, and the longest about nine seconds. It will be noticed that the augmentation appears at both rates. Figure 7 was obtained from another animal which did not show any definite increase in the knee jerk during inspiration. The rate of stimulation varied between one every two seconds to one every seven seconds. With the exception of the slight irregularity in the region marked *x*, but which soon

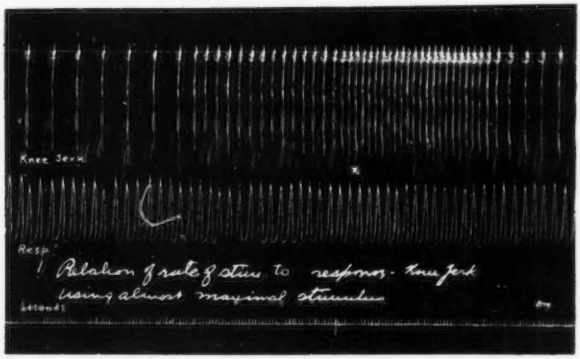

Fig. 7. Knee jerk—barbitalized dog. The relation between the rate of strong stimulation and the height of response. No evidence of a refractory state lasting as long as two seconds.

disappeared, there is no evidence of the existence of a long refractory state, since the responses are of the same magnitude at all rates of stimulation.

Inasmuch as these results differ from those obtained by Strughold, an attempt was made to discover the reason for the difference. All our earlier experiments on man were made with the subject in the supine position, the knee supported on a triangle at an angle of about 110 degrees, an angle found quite suitable for eliciting the knee jerk. Strughold's subjects were tested in the sitting position. Consequently, a series of tests was made on men in the sitting position, using a variety of methods of stimulation and recording, including a duplication of those used by Strughold. The results of these experiments were no different from those obtained by the earlier technique.

On the basis of our results, the inspiratory augmentation cannot be con-

nected with a refractory state in the true sense of the term. While our primary interest has not been concerned with the question of the refractory period, and not enough work has been done to warrant its full discussion, we may call attention to the fact that a refractory state lasting seconds is unknown for any of the tangible components of the reflex arc. Sherrington and Sowton (13) demonstrated that the absolute refractory period for an ipsilateral flexion reflex is not essentially different from that of a muscle nerve preparation, and it is not likely that the relative period is much longer. It is also not easily conceivable that the refractory periods of the central components of the reflex arc could be summed, consequently the true refractory period of any reflex arc should be the longest period of any of its components. If the diminished mean height of the knee jerk, on rapid stimulation as found by Strughold, is the consequence of a long total refractory period, the first response of the series should be the maximal height obtained for the given strength of stimulation. Whether this was true or not, we are unable to say, inasmuch as none of his published records show the initial response.

Regardless of differences in viewpoint, our results bear with those of Strughold a common interpretation. The inspiratory augmentation of the knee jerk may appear under any condition of submaximal response, regardless of whether this obtains because of submaximal stimulation, or because of a state produced within the central nervous system incident to previous activity. Both Hoffmann (14) and Strughold state that the refractory period of a reflex arc is shortened by reinforcement. Fundamentally, the shortening of the recovery period, the increase in penetrability, and perhaps excitability may be the same kind of process. Consequently, respiratory impulses which have a reinforcing effect, by shortening the refractory period, may, if the rate of stimulation is rapid enough, contribute to the demonstration of a respiratory rhythm in reflex responses. It is well known that in a small percentage of apparently normal subjects, the knee jerk is not elicitable without some special reinforcement, such as the Jendrassik hand clasp. Only in the most general sense can the term refractory be applied in these cases, and the effect of the reinforcement is not that of hastening the recovery from previous activity, but rather that of improving conductivity or increasing the excitability above that of the resting state. It is our concept that this is the essential effect of inspiratory irradiation, and that the existence of a true refractory state is not necessary for the manifestation of an inspiratory augmentation of the knee jerk.

Nature of the augmentation. The changes which presumably could bring about an increase in the extension of the leg, quadriceps thickening, and muscle action current may be classified under three heads: first, more motor neurones and consequently more muscle fibers may be thrown into action; second, no change in the number of contractile units involved but a

closer synchronization of their contraction; and third, an increase in the number, and a summation of contractions due to repetitive discharge. It is conceivable that one might be confronted with various combinations of these three. In order to throw light on the analysis of these possibilities synchronous isometric contractions and action currents were recorded. The duration of the action current, the angle of ascent of the isometric contraction, and the time from the beginning of the electrical response to the angle were taken into consideration.[2]

The results of these studies show no change in time relations which are significant, and therefore we conclude that the augmentation consists in the involvement of more contractile units, with no changes in synchronization, and no change in repetitive discharge, if such exists.

Mechanism of irradiation. The evidence presented in the preceding paragraphs indicates very definitely that the inspiratory augmentation of the knee jerk depends upon the discharge of impulses from the respiratory center. Two possibilities present themselves in the explanation of this fact. The impulses may spill over and irradiate down the cord, and in the manner indicated earlier, result in the augmentation of reflexes elicited at the right moment, or they may merely irradiate to nearby centers and through these affect the lower spinal arcs. The fact that the augmentation is demonstrable in animals decerebrated by the Sherrington (15) method clearly indicates that certain structures in the anterior neuraxis are not essential. The final decision relative to the exact mechanism through which respiratory impulses affect the knee jerk and the Achilles reflex must await further studies, including progressive ablation of the higher levels of the central nervous system, and also an inquiry into the peripheral changes incident to respiration.

In the presentation of data no special attention has been given to the Achilles jerk. Early in our work this reflex was studied along with the knee jerk in each subject. In the main, the results with the two reflexes were so similar that further extensive studies on the Achilles reaction were discontinued.

SUMMARY

1. We have presented data which demonstrate that with each inspiration in normal breathing the knee jerk and the Achilles reflex are augmented.

2. The augmentation is not demonstrable in all subjects and animals, nor in the same one at all times. The conditions, favorable and unfavorable, are discussed.

3. The phenomenon is not a reflex from the respiratory tract or muscles,

[2] Recently Cooper and Eccles (16) have shown that the "angle" is an artifact, not appearing if a frictionless lever is used. The "angle", however, has relative value.

but is dependent upon the discharge of impulses from the respiratory center.

4. There is no increase in the knee jerk during normal expiration, but during forced breathing there is some evidence of a slight increase. By forcing the respiratory effort the effects of inspiration are increased.

5. The inspiratory augmentation is demonstrable through the whole range of intensity of stimulation, from threshold to maximal, but is best shown in the weak and medium range.

6. Within the limits tested, the augmentation bears no relation to the rate of stimulation. The existence of a true refractory state is not essential for its production.

7. The inspiratory augmentation of the reflexes studied is attributed to the irradiation of impulses from the respiratory center. The exact mechanism involved is not clear, but the possibilities have been stated, and the lines of work necessary for further clarification pointed out.

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STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN BIRDS

XXVIII. Extirpation of Thymus and Bursa in Pigeons with a Consideration of the Failure of Thymectomy to Reveal Thymus Function

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The thymus remains the "enigmatic organ" of vertebrate animals. Approached by several methods of investigation the thymus has revealed some good evidence of endocrine and other function; but it is a most singular fact that the existence of no such function has been definitely found, nor even clearly confirmed, by the *removal* of the organ. It is this astonishing situation, rather than the thymus itself, which perhaps now most deserves attention and explanation. If it should turn out that the discrete structure which we call the thymus contains only a part of the tissue possessing thymic function, then thymectomy may be without effects simply because this scattered and undisturbed tissue continues to function; or indeed because it undergoes hyperplasia—a process especially prominent in the thymus itself—with early restoration of full thymic function.

In the case of birds there is considerable evidence that another organ which is of very large size in the young animal, the bursa Fabricius, is the functional equivalent of the thymus. Jolly (1915) considers the bursa "a cloacal thymus," and his view has received support from the extremely close parallelism which Riddle and Frey (1925) and Riddle (1928) found in the growth and involution of thymus and bursa in doves and pigeons; and also from the fact, definitely established for the fowl by Jolly and Pézard (1928), that castration delays the involution of the bursa in quite the same way that it is well known to delay the involution of the thymus. Further, Terni (1927, 1928) has recently indicated that some "thymus-like tissue" is to be found in the two postbranchial bodies of fowls, and Greenwood (personal demonstration to us) has observed true thymic tissue even within the capsule of a fowl's thyroid. Finally, there are the widely scattered

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lymphoid tissues which to one or another extent probably share with the thymus truly thymic functions. Though the bursa Fabricius is apparently confined to birds, the other structures named are rather probable supplements to thymic function in mammals and other vertebrates. Several recent reports on thymectomy completely fail to recognize the existence of any such supplementary or vicariously functioning tissue in any animal, and the present paper would be necessary if it considered only the probable reason for the absence of effects following simple thymectomy.

From 17 young pigeons we have removed all of the discoverable discrete thymus, and all of the bursa Fabricius, and permitted the surviving birds to live thereafter to or much beyond sexual maturity (3–16 months after operation). Though this number of survivors is not large we can satisfactorily show that this double operation is also without measurable effect on the later body growth, health, age at sexual maturity, reproduction and weights of certain endocrine organs of our animals.

MATERIAL AND METHODS. Young common pigeons, aged approximately 60 days (42 days after hatching) at the first operation, were used. Our plan was to remove both bursa and thymus at a time when both are quite large, and to follow this 3 or 4 weeks later with a second operation (or examination of the site of the organs) at the time both organs reach their maximum size (2.8 months from beginning of embryonic development) and begin their involution (see Riddle, 1928). After 12 such reoperations these were discontinued since 6 birds died within 2 to 21 days thereafter, and because thymus or bursa tissue had been found in only 4 of these 12 reoperations. Two (of 34) birds died during the first operation, and 6 died within 1 to 7 days following it. None of 12 birds died during reoperation, but 6 died at intervals of 2 to 21 days thereafter. Some of the several deaths were from uremia, following the accidental cutting and closure of the ureters (see Riddle, 1930) during bursectomy, and two were from peritonitis following rupture of the cloaca; other deaths were from hemorrhage, and still others (digestive disorder) apparently resulted from extensive interference with the innervation and blood supply to the crop incident to the removal of the long, large and double thymi of these birds. Local anesthesia was used. Twenty operated birds were reared to or beyond maturity, but only 17 of these proved at necropsy to be free of discoverable bursa and thymus tissue. In the 3 incomplete operations we found 105 mgm. of bursa in one bird, 358 and 22 mgm. thymus in the other two birds.

The bursa was removed by the method of Riddle and Tange (1928) and the thymus by a method apparently hitherto unused. In order to minimize the chances of thymus regeneration from scattered cells possibly carried on shears or forceps the actual dissection of the thymus was done without the use of these instruments. After making a median longitudinal slit from thorax to mandible the dissection was done entirely with the thumbs and thumb nails. In this way it was usually possible to loosen and extirpate an entire chain of thymus lobules without anywhere severing or perforating its connective tissue capsule. At the first operation the average amount of tissue removed from each bird was as follows: From (8) males, thymus = 0.814 gram, bursa = 0.401 gram; from (12) females, thymus = 0.554 gram, bursa = 0.452 gram. For the removal of both thymus and bursa we here introduce and use the term "thymobursectomy."

As a control we used the 15 brothers of the 7 completely operated males, and for the 10 successfully operated females we used their 26 sisters which lived to or beyond

maturity. The control birds were not hatched and reared at the same dates as their operated brothers or sisters, but all were given identical living conditions and treatment except for the operation.

EXPERIMENTAL RESULTS. *Effects on body weight.* Already at 42 days after hatching the pigeon has attained a high proportion of its ultimate body weight, and thymobursectomy at this stage is by no means an adequate test of the relation which these glands may bear to total body growth. During several succeeding months, however, these birds slowly increase in weight, and the result obtained is of some interest. The 7 operated males added 13.4 per cent of their final weight after operation, and when killed their weight averaged 99.9 per cent of that of their 15 brothers; the 10 operated females later added 14.0 per cent of their final weight, and at necropsy averaged 99.8 per cent of the final weight of their 26 unoperated sisters. Obviously this operation had no effect on the attainment of the final 14 per cent of the normal body weight.

The age of attainment of sexual maturity in females. Though it is of importance to know whether early thymobursectomy influences the age at which female pigeons become sexually mature (i.e., produce their first egg), this is not easy to measure with precision. A tabulation of the results could prove misleading, and it is necessary to analyze the data by means of the following textual statement. In one of the ten completely operated females the oviduct had been severed and occluded at bursectomy; this oviduct became the seat of a cyst, and egg-laying was not possible to this bird. The other 9 birds produced their first egg in life at ages ranging from 5.0 to 7.0 months, with an average at 5.7 months. This average for all their sisters was 8.4 months. These unanalyzed averages would suggest that the absence of thymus and bursa had greatly hastened the attainment of sexual maturity; it can be shown, however, that this difference rests mostly or wholly on the fact that the operated birds were all hatched from eggs produced during September–December, and that such birds clearly tend to mature at an earlier age than do birds hatched at other seasons. The full evidence for this influence of the season of hatching will be presented very soon in a paper of this series, but the following item largely covers the point chiefly involved here. If the age of maturity of the 8 operated females which had sisters hatched from eggs of September–December be compared with the *earliest* maturity found among such sisters we find that these latter show an average maturity at 5.65 months, while the 8 operated birds average 5.86 months at maturity; all of the controls hatched from eggs of September–December show an average maturity at 6.22 months. We must conclude that thymobursectomy did not measurably affect the age at which sexual maturity was attained in these birds.

Capacity of females for normal reproduction. The 9 females whose oviducts permitted the deposition of eggs were all allowed to produce a few

eggs before they were killed for complete examination. A total of 88 eggs was produced by these birds. Only 1 of these eggs was markedly thin-shelled; none was soft-shelled; 87 had efficient shells. In all or practically all cases the eggs were of normal weight. Only 5 per cent of "unpaired" eggs (i.e., one egg instead of two in a clutch) were produced by these birds. Though no attempt was made to hatch all eggs from thymobursectomized birds, 33 were hatched and produced entirely normal offspring. There was no measurable change from the expected percentage of fertility in the operated females. Some males, however, were entirely sterile because of the closure of both of their sperm ducts after accidental section at bursectomy. In these latter cases a cystic enlargement occurred in the epididymis to which such an occluded sperm duct was attached; this, however, seems to have left the size and function of the testis itself quite unaffected. The females laid normal eggs, with normal frequency, showed typical brooding (incubating) instincts, fed young normally and produced normal offspring.

Size or weight of organs and basal metabolism. The testes of the 7 completely operated males show no consistent difference in weight when compared with those of 15 unoperated brothers. The same is true of the 10 ovaries of operated females when compared with the 26 ovaries from unoperated sisters. Three cases of atrophied kidney resulted from the accidental section and closure of a ureter attending the bursectomy. In each case the opposite kidney had undergone marked functional hypertrophy, but this result is of course wholly unrelated to the loss of thymus function. No significant or consistent difference in intestinal length, or in weight of thyroid, liver or spleen, distinguished the 17 operated birds from their 41 unoperated controls. Thyroids from 13 operated birds were smaller, and only 4 were larger, than their controls; but the greater probability of the inclusion of occasional goitrous glands in the larger numbers of control birds (this found to be the case) probably accounts for this difference. Though the suprarenals were not weighed they were regularly inspected and seemed normal in size and appearance. In general, the skeleton seemed entirely normal. Only in the case of the relative proportion of crooked keel-bones can we supply a comparison on a numerical basis. Among the 17 operated birds were 3 with keel-bones (crista sterni) slightly crooked; among the 41 control were 3 slightly and 3 markedly crooked. Thus it is evident that no difference in the macroscopic appearance of the skeleton of the two groups was found.

The basal metabolism of 8 operated birds was compared with that of 8 unoperated sisters or brothers. The operated birds show an average within 3.5 per cent (below) that of the control, and no real or significant difference was therefore found in these few measurements.

Resistance to disease. Since no special tests were made on this subject

only a very general statement can be made. It should here be recalled, however, that the body weight, weight of various organs, basal metabolism, etc., have been found to be practically identical in the operated and control groups. All this speaks for generally similar conditions of health in the two groups. Ascariasis, in which infestation occurs very early (probably before the time of operation), did occur in both groups and in a fairly equivalent proportion (4:17; 14:41) within the two groups. Of more significance is the circumstance that, following the immediate effects of the operation (up to 21 days), not a single death occurred among the 17 operated birds during the 3 to 16 months which they were permitted to live; nor was any one of these birds frankly diseased at necropsy. In this important respect the health of the thymobursectomized birds was certainly as good as that of the controls—among which two diseased birds were found. Under the (good) conditions of life supplied to our animals the thymobursectomized individuals were fully equal to their control in health and appearance.

THE FAILURE OF THYMECTOMY TO INDICATE THYMUS FUNCTION. The observation that the removal of the thymus is without observable effect on the organism has sometimes led to the conclusion that the thymus has no endocrine function whatsoever. For the reason partially indicated at the beginning of this paper such a conclusion, in the special case of the thymus, is wholly unwarranted. Even the antitoxic and disease resistance function of the thymus (Barbàra, 1918; Jaffe and Marine, 1924; Jaffe, 1924 and others) receives little or no support from observations made on thymectomized animals (Tongu, 1919; Pearce and Van Allen, 1926; and others); but, for the same reason noted above, this fact is not a valid argument against the existence of this function. The removal of either thyroid, suprarenal or gonad affects the size and the time of involution of the thymus (Soli, 1909; Marine, Manley and Baumann, 1924; Greenwood, 1930; and others); but the removal of the thymus has either no effect or no consistent effect on either of those glands (MacLennan, 1908; Soli, 1909; Halnan and Marshall, 1914; Pappenheimer, 1914, 1917a; this study, and others). The simultaneous feeding of thyroid and thymus tissue has indicated that the latter partly counteracts the action of the former (Halverson, Bergeim and Hawk, 1916; Křiženecký, 1928, 1929); but thymectomy seems not to have given evidence of such an antagonism. The injection of thymus extracts has supplied evidence (in addition to evidence from other sources) that this organ contains a growth-promoting substance (Knipping and Rieder, 1924; Miyagawa and Wada, 1925; Asher, 1930); but thymectomy has usually completely failed to produce any adverse effect on body growth (Hammar, 1905; Halnan and Marshall, 1914; Pappenheimer, 1914, 1917a; Park and McClure, 1919; Allen, 1920; Katsura, 1922, Morgan and Grierson, 1930; this study). Through feeding minute

amounts of thymus to a very special group of pigeons Riddle (1924) obtained good evidence that a hormone of the thymus governs the secretion of the various egg-envelopes in the vertebrate oviduct; but thymectomy has probably yielded little or nothing to verify this function of the thymus.

No fact concerning the structure, origin or pathology of the thymus can transcend the now evident fact that though thymus functions have repeatedly been indicated by other methods of approach, such functions are not indicated nor supported by observed effects following the removal of this organ. What then is the meaning of this very general result? Surely one must suspect, as several observers before us have done, the existence of other tissues capable of performing one or another thymic function. Even in mammals certain phenomena observed in lymph glands give evidence of their peculiar and close relationship to the thymus We cite merely the similar involution of thymus and lymph glands preceding puberty, and Pappenheimer's observations on the hyperplasia of the regional lymphatic glands of the rat following thymectomy (1914) and on the essential equivalence or possible identity of the small thymic cells and the lymphocytes derived from lymph glands (1917b). Quite independently of the animal used, and of the particular thymus function involved, we consider the following conclusion justified and necessary: *The absence of effects following complete removal of the discrete thymus probably means merely that a part only of the tissue with thymic function is removed by such ablation of the thymus.*

This point of view is now especially insistent in the case of birds where the large bursa Fabricii is so prominent during the growth period, where it occasionally persists throughout life (Riddle and Frey, 1925), and where we meet all of the other important conditions mentioned in an introductory paragraph of this paper. It is therefore necessary to examine especially the work which has hitherto been reported as bearing on an endocrine function of the thymus in birds (for general literature on the thymus see Hammar, 1921; Marine, 1928).

Riddle (1924) found and studied a special group of 5 doves which exhibited the following syndrome: Deficient shell and albumen in eggs with normal yolks; frequent single instead of paired ovulations; greatly diminished or suppressed fertility and hatchability; all this from females whose reproduction had previously been normal in all these respects, and in which unexpectedly and extremely small thymi were found at necropsy. These defects of the egg-envelopes were very promptly corrected by the administration *per os* of thymus tissue—and by this tissue only. Riddle concluded that those results "demonstrate the presence in the thymus of a substance, apparently a hormone (for which the name *thymovidin* is proposed), which is indispensable to the production of the egg envelopes." In

a search of the literature for results from thymectomy which could oppose or confirm this apparent control of egg-envelope formation by thymus hormone the data of Soli (1911) were found and cited. That study seemed definitely to confirm Riddle's view that a thymus hormone controls the secretion of the egg-shell, though Soli had considered his results (soft or absent shells on the eggs of thymectomized fowls) as only a confirmation of work of others on a relation of the thymus to calcium metabolism.

In the most recent report which at all touches this topic Morgan and Grierson (1930) erroneously state that the one mature reproducing pigeon thymectomized (partially) by Riddle (1924) also at first "laid eggs with noticeably soft shells." The fact is that all of the eggs produced by this bird following thymectomy had good shells—all within limits that would permit the hatching of the young—though it was shown (data there completely tabulated) that some of those eggs had slightly thinner shells (higher rate of moisture loss) than those preceding the operation. At that time Riddle accepted Soli's report as a fairly conclusive and reliable statement of the effects of a simple thymectomy (with partial regeneration) on the formation of the egg-shell, and supposed that the slight thinning of some of the shells produced by his operated pigeon should be interpreted in the same sense. But new data, to be next described, throw some doubt on the validity of Soli's results.

It is true that the birds thymectomized by Soli were mature laying hens and that they therefore probably had little or no bursa tissue requiring removal in order to bring the amount of tissue with "thymic function" to a relatively low level The only adequate repetition of Soli's work is that of Greenwood (personal communication; to be published in Proc. Soc. Exper. Biol. and Med., 1931) who likewise removed the thymus from a few mature females (brown Leghorn) but found, contrary to Soli, that eggs and egg-envelopes produced at all later periods were quite normal. It is possible, even probable, that complete thymectomy in an immature bird delays the involution of the bursa in that bird (one of our thymobursectomized birds had no thymus but a regenerate bursa *after* sexual maturity). If this is true the studies of Ackert and Morris (1929) and Morgan and Grierson (1930) are of very limited significance to this question—since their removal of the thymus in young fowls would not necessarily mean an extensive and sudden reduction of tissue with thymic function during the period of laying, nor even at any earlier period.

It is evident therefore that the removal of the thymus of a young bird is not the equivalent of its removal from a mature bird, nor is it the equivalent of thymobursectomy performed on a young bird. Neither can the present study, in which both thymus and bursa were removed from young birds, be considered as quite definitely contradictory to Soli's results; for, the early removal of these organs may have permitted hyperplasia in the

remaining scattered tissue with thymic function before the beginning of egg-production. It must be remembered that Soli reported only a very temporary effect, and that a fairly rapid increase of tissue with thymic function following thymectomy is the plausible explanation of the temporary nature of this effect. Nevertheless, our own tests, and the much more adequate (unpublished) tests made by Greenwood, with wholly contradictory results in mature fowls, definitely question the validity of Soli's report of shell-less eggs as a result of simple thymectomy in mature fowls. It is conceivable that the particular race of fowls used by Soli is responsible for the difference in results and it is very desirable that that race (not mentioned) be tested again.

Discussion. The series of operations described here were performed (mostly late 1928) for the more special purpose of learning to what extent such processes as the building of the egg-envelopes and the age of attainment of sexual maturity would be influenced by an early removal of the thymus and bursa—the two largest aggregates of thymic tissue; secondarily, it was thought that from these young birds data of value concerning growth, organ weights and disease resistance might be obtained. It is only since our operations were performed that Terni and Greenwood have found thymus tissue in other locations in fowls, and that we have been led to suspect its still wider distribution in the body. It is also since that time that a full consideration of our results, and a more thorough examination of the literature on the subject, have led to the conclusion that thymectomy has almost everywhere and completely failed to reveal or to confirm those thymus functions which have been rather clearly indicated by various other methods of investigating this organ.

We wish to express our thanks to Miss Irene Polhemus for assistance rendered in connection with this study.

SUMMARY

Thymus and bursa Fabricii were completely extirpated (thymobursectomy) from 17 common pigeons at a first operation performed about 42 days after hatching, or at a second operation 3 to 4 weeks later; 15 brothers of the 7 operated males, and 26 sisters of the 10 operated females, served as control. Birds were reared to or beyond maturity (3–16 months after operation).

That part of the body growth (14 per cent of total) which occurred after the operation was not affected by the loss of these two organs.

No clear and consistent difference was found in weight of testes, ovaries, thyroid, liver or spleen in operated and control. No difference was observed in bone abnormalities, basal metabolism, health and appearance.

The operation had no effect on the time or age at which the females became sexually mature. The operated females produced eggs (88) whose

envelopes were quite normal, with only a single exception; there were no soft-shelled eggs. These data and the more crucial data of Greenwood strongly question Soli's report of soft-shelled eggs as a result of thymectomy in mature fowls, unless the particular race of fowl used really determines the result.

We conclude that tissue with "thymic function" is probably widely scattered in the body of vertebrate animals generally. This condition is now rather more evident in birds than in other groups. In birds such tissue is probably regularly represented in the two thymi, the bursa Fabricii, the two postbranchial bodies, in the lymph glands and perhaps in other lymphoid organs; occasionally it is undoubtedly present in still other areas adjacent to the thymus.

An extensive examination of the literature leads to the conclusion that thymectomy has almost everywhere and completely failed to reveal or to confirm those functions of the thymus which have been rather clearly indicated by various other methods of investigating this organ

The explanation of the failure of thymectomy to produce effects probably rests upon the existence and hyperplasia of other widely scattered tissue having thymic function. No result ever obtained from simple thymectomy is usable as evidence against the existence of an endocrine or other function of the thymus.

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A STUDY OF RESPONSES TO WORK ON A BICYCLE ERGOMETER[1]

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Any experimental data that give hint as to why the capacity of the individual for muscular exertion is limited is still of interest. It is generally recognized that individuals are not equal in physical ability; and that, in all probability, no two persons react in exactly the same way during the performance of a particular type and load of work.

One of the outstanding problems of the body during physical exertion is that of supplying an adequate amount of oxygen to the active muscles. In the accomplishment of this task several devices are employed, and any one of these may be called into action more at one time than at another. When the limit of ability to supply oxygen is being approached, or is reached, the failure of compensation may be due to the inadequacy of one, or more, or all of these devices.

There is a close relationship between oxygen absorption, the respiratory minute-volume, the respiratory rate, the frequency of the heart beat, the oxygen pulse and the performance of muscular work. These relationships are here investigated by means of the respiratory exchange during the performance of five loads of work on a bicycle ergometer.

EXPERIMENTAL PROCEDURE. Six men of sedentary habits repeatedly served as subjects. They were required to work on a bicycle ergometer of the Krogh pattern, where they carried loads of 2000, 4000, 6000, 8000, and 10000 foot-pounds per minute. Douglas bags were employed for the collection of the expired air and the plan of set-up was similar to that used by Campbell, Douglas and Hobson (1920). Mueller water valves were used with an effective area of 3.5 cm. The delivery pipe dipped about 2 mm. below the surface of the water and the resistance, even with a high breathing rate, was slight.

A kymograph was set up with a half-minute time marker, a signal magnet, and a key; and with a tambour connected to the subject's side of the expiratory water valve. This record was used to verify the duration of each bag collection and to count the rate of breathing. Shortly before use

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each bag was flushed out with about 10 liters of expired air and emptied as completely as possible by a standardized method of rolling up and squeezing.

Every experiment was preceded by a period of complete rest of from 15 to 20 minutes, after which the resting respiratory exchange was determined. Between the work periods a rest of 20 minutes was allowed, after which the subject then did the next larger load. Each load was carried for from 6 to 8 minutes. The data that are herein recorded are the averaged results of a number of experiments with each load of work.

The pedaling of the ergometer was always done at the rate of 70 revolutions per minute. In order to avoid marked temperature changes in the body two electric fans were played on the subject as he worked.

Crest-load. In the following discussion of data reference will frequently be made to the crest-load. This expression has been adopted from Briggs (1920) who has made a distinction between a normal-load, crest-load, and over-load of work on the basis of oxygen absorption. According to this distinction a load is normal so long as the intake of oxygen at the time of work is adequate to supply the need of the muscles, and the load is an over-load if the oxygen intake is insufficient. The crest-load is that one in which the oxygen supplying mechanisms, working at full capacity, are just able to supply the oxygen need and thus maintain an even balance between the call for and the use of oxygen. The percentage of carbon dioxide in the expired air of a work period has been employed by Briggs to determine which is the crest-load.

When exertion of increasing magnitudes is studied, it is found that the percentage of the expired carbon dioxide rises with each addition to the load of work so long as these are normal-loads. When, however, the percentage of carbon dioxide in the exhaled air begins to drop an over-load has been reached. The crest-load is the one for which the percentage of exhaled carbon dioxide is largest.

In table 1 are given the percentage and the total minute-volume of carbon dioxide eliminated by each of our subjects. The crest-load of each man is indicated by the heavy type in the percentage columns of the table.

Oxygen absorption. A considerable number of past researches have tended to establish the fact that the per minute consumption of oxygen varies almost directly with the amount of work done in the same unit of time. The data we present in table 2 in general support this observation. They, however, bring out the fact that when the load of work becomes an overload individual differences in oxygen intake appear. For easy visualization of the changes in oxygen absorption the data may be expressed in several ways. In the second part of table 2 the amount of increase in oxygen usage for each stepping up of the load is given. This method shows

at a glance that the usage of oxygen by W. C. increased in practically equal amount, an average of 564 cc., for each step of increase in the load of work up to our maximum of 10000 foot-pounds. Means and Newburgh (1915) have shown that the linear relationship between oxygen absorption and work is more clearly brought out by expressing the increased usage of oxygen in per cent of the resting value of oxygen consumption.

Our data clearly bring out the fact that with moderate loads of work the adding of equal increments to the load results in approximately equal in-

TABLE 1

The elimination of carbon dioxide during work

SUBJECT	PERCENTAGE CONTENT OF CO_2 IN EXPIRED AIR						THE OUTPUT OF CO_2 PER MINUTE IN CUBIC CENTIMETERS					
	Rest	2,000 foot-lbs.	4,000 foot-lbs.	6,000 foot-lbs.	8,000 foot-lbs.	10,000 foot-lbs.	Rest	2,000 foot-lbs.	4,000 foot-lbs.	6,000 foot-lbs.	8,000 foot-lbs.	10,000 foot-lbs.
W. C.	2.99	4.11	4.41	**4.44**	4.17	3.88	181	650	1,266	1,925	2,188	2,838
L. H.	4.08	4.69	**5.96**	5.34	4.87	3.26	219	734	1,518	2,327	2,683	2,905
D. M.	2.25	3.07	3.98	**4.68**	4.57	4.19	224	795	1,637	1,820	2,348	2,713
H. M.	3.60	4.28	**5.16**	**5.16**	4.48		236	843	1,505	1,948	2,621	
R. J.	3.23	4.08	4.49	**4.65**	4.50	4.12	253	744	1,458	1,692	2,533	3,761
S. Y.	3.01	3.80	**4.06**	3.97			224	864	1,489	1,874		

TABLE 2

SUBJECT	CUBIC CENTIMETERS OF OXYGEN ABSORBED PER MINUTE DURING WORK						THE INCREASE IN OXYGEN CONSUMPTION PER INCREASE IN LOAD				
	Rest	2,000 foot-lbs.	4,000 foot-lbs.	6,000 foot-lbs.	8,000 foot-lbs.	10,000 foot-lbs.	2,000 foot-lbs.	4,000 foot-lbs.	6,000 foot-lbs.	8,000 foot-lbs.	10,000 foot-lbs.
W. C.	261	829	1,413	1,924	2,513	3,082	568	584	511	589	569
L. H.	278	832	1,425	1,972	2,422	2,423	554	593	547	450	1
D. M.	267	911	1,530	2,176	2,479	2,608	644	619	646	303	129
H. M.	282	945	1,584	1,990	2,356		663	639	406	366	
R. J.	303	911	1,487	1,844	2,430	3,124	608	576	357	586	694
S. Y.	271	918	1,426	1,837			647	508	411		

crements in the absorption of oxygen. Means and Newburgh established this relationship for loads up to 6000 foot-pounds (800 kilometers). With heavier loads, however, the amount of oxygen consumed does not necessarily come up to expectation. This is contrary to Studer's (1926) experience in horizontal locomotion. He found that for speeds of 2 to 4.5 kilometers per hour the oxygen consumption is proportional to the speed, but that above 4.5 kilometers the consumption is relatively greater. Variation in speed no doubt accounts for the difference. In our experiments a constant speed was employed with all loads of work. Of our six

subjects, W. C. and R. J. maintained the linear relationship of oxygen to work up to and including 10000 foot-pounds.

A falling off in oxygen consumption was conspicuous in our other subjects soon after the crest-load was reached. Thus L. H. maintained the linear relationship up to 6000 foot-pounds, in which the increase in oxygen consumption was about 550 cc. for each addition of 2000 foot-pounds to the load of work. When the load was next stepped up to 8000 foot-pounds, his oxygen intake rose only 450 cc.; and when the load was later increased to 10000 foot-pounds, the oxygen intake failed entirely to augment; hence L. H. maintained the linear relationship for only one step beyond his crest-load. D. M., with a regular average increase per load of 645 cc. up to 6000 foot-pounds, augmented his oxygen usage for the next two upward steps by only 303 and 129 cc. respectively. With him the linear relationship was broken for all over-loads. For H. M. the linear relationship fell off for loads above 4000 foot-pounds, which was his crest-load; while for S. Y. a linear relationship could only be established for exceedingly moderate loads. With a load of 4000 foot-pounds S.Y's oxygen absorption was already as much as 100 cc. below the predicted intake.

It is evident that we succeeded in pushing four of our six subjects to a load of work sufficiently high to break the law of linear relationship between load and absorption of oxygen. Furthermore, in each case that the break occurred the absorption of oxygen for the heavier loads of work fell under the theoretical or expected value. It is also evident that additions to the load of work may be made for which the body can not further increase its intake of oxygen.

The frequency of the heart beat. Most students of this problem have compared the pulse frequency with the amount of oxygen absorbed during work. Boothby (1915) showed practically a linear relationship between the pulse frequency and oxygen consumption up to an absorption of 1000 cc. of oxygen. Means (1924) expresses the relationship between pulse frequency and load of work as a curve which is quite flat for moderate loads and tends to rise more sharply with loads above 4500 foot-pounds.

We have counted the pulse of five of our six subjects and give the average counts in table 3. As the results are more easily interpreted when expressed in terms of work accomplished, we confine our attention to an analysis of the relationship of pulse frequency to load of work. The data in table 3 clearly support the linear relationship and fail to substantiate the curve relationship of the frequency of heart beat and load of work. All of the five subjects show that at least a rough degree of parallelism may be maintained between these two factors up to some particular load, which is found to vary with each man. R. J. and H. M. maintained the linear relationship for all loads. With each addition of 2000 foot-pounds to the load of work, the successive increases in pulse count for R. J. up to 10,000

foot-pounds were 30, 27, 22, 23 and 21 respectively; and for H. M. up to 8000 foot-pounds were 23, 31, 24 and 26 respectively.

The remaining three subjects showed that a break eventually occurs in the linear relationship. W. C. experienced an acceleration of approximately 29 beats in his heart rate with each increase of 2000 foot-pounds in the load of work up to that of 8000 foot-pounds, but with the stepping up of the load to 10,000 foot-pounds the heart failed to keep pace with the demand made upon it and only advanced its rate by 15 beats. L. H. likewise maintained the linear relationship up to a load of 8000 foot-pounds. Then after having had an average augmentation of 25 beats of the heart with the successive equal additions to the load of work, the increase in the frequency of the pulse was only six beats when the load was stepped up from 8000 to 10,000 foot-pounds. D. M.'s heart responded in the usual way up to a load of 4000 foot-pounds, with an increase of 40 or more beats per step; but seemed thereafter incapable of a material further acceleration.

TABLE 3

SUBJECT	PULSE RATE PER MINUTE						THE PULSE RATE INCREASE				
	Rest	2,000 foot-lbs.	4,000 foot-lbs.	6,000 foot-lbs.	8,000 foot-lbs.	10,000 foot-lbs.	2,000 foot-lbs.	4,000 foot-lbs.	6,000 foot-lbs.	8,000 foot-lbs.	10,000 foot-lbs.
W. C.	74	98	127	156	187	202	24	29	29	31	15
L. H.	86	106	144	168	189	195	20	38	24	21	6
D. M.	89	135	174	174	179	180	46	39	0	5	1
H. M.	78	101	132	156	182		23	31	24	26	
R. J.	75	105	132	154	177	198	30	27	22	23	21

There was no increase in the pulse frequency when the load was stepped up from 4000 to 6000 foot-pounds, it was accelerated five beats when the load was increased to 8000 foot-pounds, and only one beat when the load was finally increased to 10,000 foot-pounds.

From the above data it appears that the frequency of the heart beat will maintain an approximate linear relationship with the demands of physical work up to a certain load, which varies with the individual; and that with the addition of heavier loads beyond this point the heart will respond in a lesser degree than theretofore. It is also evident that eventually a load of work may be undertaken to which the heart is unable to respond with any further increase in frequency of beating.

The oxygen pulse. Henderson and Prince (1914) define this as the amount of oxygen consumed by the body from the blood of one systolic discharge of the heart. Its value is calculated by dividing the amount of oxygen that is absorbed by the individual per minute by the number of heart beats in a minute. These authors found that the oxygen pulse is

subject to minor irregularities; and that, starting with the slowest heart rates and lowest figures for oxygen absorption, a slight exertion which accelerates the pulse to 80 or 100 per minute results in an oxygen pulse that remains nearly uniform. From this point on the oxygen pulse increases rapidly with physical exertion. A professional bicycle rider observed by Benedict and Cathcart (1913) reached his maximal oxygen pulse, 16.8 cc., when working at a rate that caused an oxygen absorption of 1700 cc. and a pulse frequency of 163 per minute. Henderson and Prince conclude that the oxygen pulse increases rapidly with acceleration of the heart, and in most cases this reaches its maximal value of 11 to 17 cc. at heart rates of 130 to 140 beats per minute. With further acceleration of the heart the oxygen pulse may even tend to decrease.

Boothby (1915) finds that, for work requiring up to 1000 cc. of oxygen a minute, the oxygen pulse maintains a definite relationship to the amount of oxygen absorbed. This is expressed in a gradually ascending curve rather than in a straight line.

TABLE 4

Oxygen pulse

SUBJECT	REST	2,000 FOOT-POUNDS	4,000 FOOT-POUNDS	6,000 FOOT-POUNDS	8,000 FOOT-POUNDS	10,000 FOOT-POUNDS
W. C.	3.5	8.5	11.1	12.3	13.4	15.3
L. H.	3.2	7.8	9.9	11.7	12.8	12.4
D. M.	3.0	6.9	8.8	12.4	13.8	14.4
H. M.	3.6	9.4	12.0	12.8	12.9	
R. J.	4.0	8.7	11.3	12.0	13.7	15.8

A summary of the oxygen pulse data of our five cases is given in table 4. The averages of these data for all loads of work up to 8000 foot-pounds (2440 cc. oxygen consumption) give a plotted curve with the concavity toward the abscissa similar to that described by Boothby. The curves for the individual cases are not as smooth as the one figured by Boothby. W. C. and R. J. each give similar curves which follow the ideal curve up to a load of 8000 foot-pounds. With the next step in the load up to 10,000 foot-pounds the oxygen pulse curve of each then makes a sudden upward bend from the general trend of the curve. These two men therefore increase their oxygen pulse for all loads of work up to that of 10,000 foot-pounds. The oxygen pulse curve of D. M. rises reasonably like the ideal curve with all loads of work up to 10,000 foot-pounds. L. H., on the other hand, after showing an increase in the oxygen pulse for all loads up to 8000 foot-pounds failed with the heaviest load, 10,000 foot-pounds, to make a further increase, and instead showed a decrease. The oxygen pulse curve of H. M. rises more rapidly than the others and tends to flatten out

for loads above 4000 foot-pounds. Beginning with a resting oxygen pulse of 3.6 cc. this rises with increasing loads of work to 12 cc. at 4000 foot-pounds, 12.8 cc. at 6000, and only 12.9 cc. at 8000 foot-pounds.

PULMONARY VENTILATION. *Minute volume of breathing.* The observations of Lindhard and others indicate that the ventilation of the lungs during physical exertion is practically always sufficient to allow the blood to become fully saturated with oxygen during its passage through the lungs. There seems to be no evidence in normal individuals that the respiratory mechanism fails to provide an adequate ventilation of the lungs. Hence it is to be expected that under the requirements of moderate exertion a definite relationship will be maintained between the minute-volume of breathing and the oxygen consumption. It has been the custom to show the correlation between these (Lindhard 1915, Boothby 1915).

In order to conform with the rest of our analyses we shall correlate the respiratory changes of exertion with the load of work accomplished per minute. In table 5 appear the averaged data for the minute volume and frequency of breathing for equal increments of work up to 10,000 foot-pounds. It will be observed that four of the six subjects maintain a linear relationship between the minute-volume of breathing and the load of work up to at least the crest-load of each individual.

The ideal linear relationship between the ventilation of the lungs and load of work may for convenience be visualized as follows: 8, 22, 36, 50, 64, and 78 liters respectively for rest, 2000, 4000, 6000, 8000, and 10,000 foot-pounds. Judged by this standard W. C. (see table 5) maintained a good linear relationship up to a load of 8000 foot-pounds. Up to 6000 foot-pounds his pulmonary ventilation line is slightly under the standard, while at 8000 foot-pounds it passes slightly above. In the step from 8000 to 10,000 foot-pounds the breathing becomes still more excessive. This was to be expected as his crest-load was determined to be 6000 foot-pounds. L. H. shows a good linear relationship up to 4000 foot-pounds and then, according to the standard, superventilates his lungs during the periods of work with the next higher loads. With a load of 10,000 foot-pounds his excess in lung ventilation reaches 23 liters. The crest-load for L. H. was 4000 foot-pounds. H. M., whose crest-load is 6000 foot-pounds, maintained a good ventilation linear relationship with his load of work up to that point; while with a load of 8000 foot-pounds he had a moderate excess of breathing of a little more than five liters. R. J. maintained a good linear relationship up to 6000 foot-pounds, his crest-load; but with larger loads of work showed an extraordinary superventilation.

D. M. and S. Y. invariably showed a hyperventilation of the lungs with moderate loads of work, which made them poor subjects for this phase of our study.

The evidence presented above supports the usual conclusion of a linear relationship between pulmonary ventilation and the degree of physical exertion. It further shows that superventilation of the lungs begins in most men soon after an over-load has been reached.

Frequency of breathing. The number of breaths per minute increases roughly proportional to the load of work up to and slightly beyond the crest-load. For four of our six subjects this was true. These data have been assembled in table 5. H. M. maintained a rather good linear relationship between the frequency of breathing and the size of the load of

TABLE 5

SUBJECT	MINUTE-VOLUME OF BREATHING IN LITERS						FREQUENCY OF BREATHING PER MINUTE					
	Rest	2,000 foot-lbs.	4,000 foot-lbs.	6,000 foot-lbs.	8,000 foot-lbs.	10,000 foot-lbs.	Rest	2,000 foot-lbs.	4,000 foot-lbs.	6,000 foot-lbs.	8,000 foot-lbs.	10,000 foot-lbs.
W. C.	7.32	19.29	33.10	49.80	65.75	84.91	11.3	16.8	20.6	22.9	25.3	38.0
L. H.	7.21	17.05	30.37	53.03	73.41	100.74	9.7	13.1	15.0	18.3	30.3	52.4
D. M.	11.36	30.92	41.43	48.93	58.46	72.21	34.6	62.3	54.8	35.5	37.1	41.3
H. M.	7.97	21.12	36.67	48.85	69.04		15.7	20.8	24.7	27.6	31.0	
R. J.	8.73	20.18	35.83	48.83	77.13	102.59	14.2	15.7	21.6	23.5	34.7	43.3
S. Y.	8.37	28.09	41.19	59.00			19.2	37.0	40.0	42.0		

TABLE 6

Volume per breath

SUBJECT	REST	2,000 FOOT-POUNDS	4,000 FOOT-POUNDS	6,000 FOOT-POUNDS	8,000 FOOT-POUNDS	10,000 FOOT-POUNDS
W. C.	648	1,148	1,607	2,175	2,598	2,234
L. H.	743	1,302	2,025	2,891	2,423	1,923
D. M.	328	496	756	1,378	1,575	1,748
H. M.	508	1,015	1,485	1,770	2,227	
R. J.	615	1,285	1,659	2,078	2,223	2,369
S. Y.	436	759	1,030	1,405		

work up to the largest load he could be induced to carry, 8000 foot-pounds. W. C., reaching his crest-load at 6000 foot-pounds, maintained roughly the linear relationship to 8000 foot-pounds; but, with the next step up to 10,000 foot-pounds, accelerated the rate of breathing enormously, from 25.3 to 38 breaths a minute. R. J. very roughly maintained the correlation of frequency to load to 6000 foot-pounds, his crest-load; and thereafter augmented the number of breaths per minute so rapidly that with a load of 10,000 foot-pounds he breathed 43.3 times a minute. L. H. continued the linear relationship to 6000 foot-pounds—2000 foot-pounds beyond his crest-load, after which he also accelerated his rate enormously. D. M.

and S. Y. seemed unable to keep from breathing somewhat in rhythm with the rate at which they pedaled the ergometer; hence the data for them do not conform with the normal frequency of breathing response.

The depth of breathing. Since it is the amount of air actually reaching the alveoli of the lungs which counts as far as oxygen absorption is concerned, it is interesting to learn how much fresh air different men get past the dead space during exertion. While our data do not make it possible to estimate the exact extent of alveolar ventilation they do give the volume of air breathed per breath and from this we may pass judgment on the efficiency of alveolar ventilation.

Plotted curves of the data in table 6 bring out a well defined linear relationship for the depth of breathing to load of work for moderate exertion. L. H. and W. C. show an excellent linear type of increase in the volume per breath up to loads of 6000 and 8000 foot-pounds respectively; while R. J., S. Y. and H. M. maintain somewhat roughly the linear relationship to loads of 6000 and 8000 foot-pounds. For any stepping up of the load beyond the maxima indicated the rate of increase in the depth of breathing falls off and a load may eventually be reached that marks the turning point after which the volume of air per breath is reduced. This reduction in volume per breath occurred with W. C. after the 8000 foot-pound load, when he inhaled 2598 cc. of air per breath; while with a load of 10,000 foot-pounds the volume was only 2234 cc. per breath. L. H. reached his maximum depth of breathing with a load of 6000 foot-pounds, when the volume was 2891 cc. per breath. With a load of 8000 foot-pounds it was reduced to 2423 cc.; and with one of 10,000 foot-pounds, to 1923 cc. per breath.

A comparison of the compensatory factors. In view of the discussion as to what it is that limits voluntary physical effort it is of interest to examine more fully the data of some of our subjects. The circulation is usually looked upon as the more important factor in the limitation of muscular work. Hill, Long and Lupton (1925) have said, "The chief determining factor, therefore, in the oxygen intake is the rate of circulation of the blood. Large differences probably exist between different individuals in respect to the outputs of their hearts. Some individuals can naturally run, or walk up hill, for long periods without distress; others, able-bodied with no physical or nervous defect, soon suffer from dyspnoea when they attempt to do so. This may be partly a matter of the diffusion constant of the lungs for oxygen; largely, however, it is probably one of the capacity of the heart itself." Clark-Kennedy (1926) and his co-workers question this conception of the limitation of physical effort and conclude that "voluntary effort is limited by failure of the functional capacity of the cardio-respiratory system as a whole, not by premature failure of any one member of

this team." They believe that a rise in hydrogen ions is probably important in causing the subjective distress which limits voluntary effort.

Among our subjects W. C. made the best compensation to the demands of exertion. He augmented his absorption of oxygen in equal amounts with each successive equal increase in the load of work up to and including 10,000 foot-pounds. He alone had a respiratory quotient below unity while carrying a load of 10,000 foot-pounds, which indicates that his output of lactic acid into the blood stream had not then become excessive. Yet his data give evidence that he had practically reached the limit of voluntary effort. In the last step up of the load of work his heart failed to accelerate its rate of beating proportionately with the increase in load. His pulse rate accelerated only half as many beats as it had for other equal steps in the load of work. In spite of this failure on the part of the heart he met the demand for oxygen by increasing the coefficient of utilization of oxygen; that is, as shown by the increase in the oxygen pulse, he withdrew a larger amount of oxygen from a unit volume of blood. His breathing with the 10,000 foot-pound load was only moderately above that demanded of the linear correlation of minute-volume and load. Had W.C. been pushed to carry a heavier load than that of 10,000 foot-pounds it seems likely that his heart would have been the first factor to fail in the task of supplying oxygen to the tissues.

L. H. made, while carrying a load of 10,000 foot-pounds, an ineffective attempt to meet the need of oxygen by, what was for him, an extraordinary augmentation in breathing. In spite of this large increase in pulmonary ventilation the absorption of oxygen remained the same as it had been with a load of 8000 foot-pounds. Seemingly two compensatory factors failed to respond adequately to the demands made upon them. In the first place the heart failed to accelerate as much as is required by the linear relationship between frequency of beat and load of work. An acceleration of something over 20 beats per minute was to be expected; instead the acceleration was only 6 beats. In the second place the discharge of oxygen from the blood to tissue was not as favorable as in other successful workers. His oxygen pulse, instead of increasing, fell off slightly, from 12.8 to 12.4 cc. The respiratory quotient was above 1 for loads above 2000 foot-pounds; thus an extraordinary discharge of lactic acid into the blood stream is indicated. In the case of L. H., therefore, the failure to obtain the theoretical absorption of oxygen when a load of 10,000 foot-pounds was carried must be attributed to the inability of the heart to meet the demands made upon it; and to an inadequate unloading of oxygen from the blood as it passed through the tissue capillaries.

While D.M.'s breathing during physical exertion was anomalous, the data for him bring out interesting relationships. D. M. maintained the theoretical relationship between the absorption of oxygen and load of

work only up to a load of 6000 foot-pounds, during the exertion of the next two larger loads the augmentation of oxygen intake fell decidedly below the theoretical value. His breathing, with the heavier loads of work, was somewhat subnormal; while the pulse frequency practically failed to further accelerate after a load of 4000 foot-pounds was reached. The oxygen pulse rose somewhat with each upward step of the load of work; thus indicating that the coefficient of utilization of oxygen increased steadily with the increase in the load of work. This subject, it may be assumed, was limited in working capacity by the failure of the functional capacity of both members of the cardio-respiratory team.

H. M. was unwilling to carry a load of 10,000 foot-pounds; hence some important data needed for our comparison are not available. H. M.'s crest-load was around 4000 foot-pounds. His absorption of oxygen increased normally up to this load, but fell below expectation for the next two larger loads. Just why this was so is not clearly evident from the data at hand. Pulmonary ventilation increased normally and the pulse frequency accelerated normally in linear relationship with the load even up to that of 8000 foot-pounds. A hint of inadequacy is found in the oxygen pulse, which failed to increase normally for loads above 4000 foot-pounds.

During the exertion of carrying the heavier loads these subjects gave evidence of failure in compensation. In two of the cases the heart seemed to be the first of the coöperative mechanisms that failed to keep up with the demands of the occasion. In two instances the unloading of oxygen from the blood did not augment as it did in the two men who made the best compensations for a load of 10,000 foot-pounds. In only one case was there any likelihood that the respiration failed to respond to the demand made upon it.

SUMMARY

A linear relationship between the amount of oxygen absorbed and the load of work was maintained by all subjects during moderate exertion. As the load was stepped up by equal increments in four of six cases, the linear relationship was broken soon after the crest-load was passed. In each of these the absorption of oxygen was then less than that of the expected or theoretical requirement. An over-load was attempted by one man in whom the oxygen supplying mechanisms were unable to further increase the oxygen intake.

The pulse frequency also, like oxygen absorption, augments roughly in a linear relationship to the increase in work up to a certain load which is found to vary from man to man. Beyond this the response to additions to the load is in lesser degree than theretofore. Finally a load of work may

be accomplished to which the heart is unable to respond with further increase in frequency of beating.

The oxygen-pulse rises steadily with an increasing load of physical work, and gives a plotted curve with the concavity towards the abscissa, except for the heaviest loads. Some individuals fail entirely or make but little addition to the oxygen pulse as their limit of load carrying ability is approached. Good reactors, on the other hand, increase the oxygen pulse beyond expectation when carrying the heaviest loads.

The minute-volume of breathing for the four subjects who reacted well maintained a linear relationship to the load of work up to the crest-load. For larger loads the increase in lung ventilation becomes excessive and diverges upward from the normal line of augmentation.

There is a tendency for the frequency of breathing to increase proportionately with the load up to or just above the crest-load. With overloads of work the frequency of breathing augments inordinately. This means that the depth of breathing likewise increases more or less proportionately up to the same load. Above this load, in some men, the depth of breathing continues to grow deeper; but, in others, the frequency of breathing becomes more rapid at the expense of the depth.

The limit of voluntary physical effort in two cases seemed to be due primarily to failure of the heart to augment proportionately to the increased demands of the tissues for oxygen. Another limiting factor was indicated in the failure of the oxygen-pulse to augment in the usual degree. This is interpreted to be due to an inability to adequately step up the coefficient of utilization of oxygen.

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THE SENSITIZATION OF VASCULAR RESPONSE TO "SYMPATHIN" BY COCAINE AND THE QUANTITATION OF "SYMPATHIN" IN TERMS OF ADRENALIN

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Newton, Zwemer and Cannon (1931) and Cannon and Bacq (1931) have recently shown that an adrenin-like hormone which they called "sympathin" is set free by action of sympathetic nerve impulses on smooth muscle. The tests in these observations were made on denervated organs. It was necessary, however, to wait several days after denervation for the organs to become sensitized. Elimination of this delay is desirable. We decided, therefore, to try to dispense with the sensitization period by the use of certain drugs—sodium bicarbonate, glycocoll, calcium ions and cocaine—which have been reported as reinforcing the action of adrenalin under certain circumstances. As an indicator we employed blood pressure. By means of it we have attempted to quantitate sympathin in terms of adrenalin.

METHOD. It is desirable to use young vigorous male cats weighing between 3 and 4 kgm. Elliott's preparation (1912), with a few modifications, was used. The animals are decerebrated without anesthesia, as described by Cannon and Britton (1925). With one blow of a hammer a sharp instrument is driven into the cranial cavity through the squamous portion of the temporal bone above the external auditory meatus, and then with one stroke the cerebral peduncles are severed. Decerebrate rigidity appears instantly. The animal will survive in this condition for several hours. Normal breathing is not hindered if the operation is properly executed and serious hemorrhage is avoided.

A tracheal cannula for artificial respiration after pithing, a carotid cannula for the blood-pressure tracing and a femoral vein cannula for intravenous injection are next inserted. The abdomen is then opened and the sympathetic chains cut at the third lumbar ganglion, to avoid stimulating the lower portion during the pithing of the spinal cord. A pair of fine shielded electrodes is applied to the sympathetic chains below the point sectioned, usually just above the promontory of the sacrum.

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In case it is necessary to occlude the circulation in the hind end of the animal, a heavy thread is passed under the aorta and vena cava immediately behind the renal vessels and the ends of the thread brought up through a thick glass tube with edges rounded to avoid injury of the artery and vein.

In a few cases both adrenal glands were ligated at this stage.

By this procedure the abdomen is opened only once and there is minimal handling of the intestines so that the blood pressure is not lowered.

In order to obtain a satisfactory preparation, it is important to carry on all operations on the animal before pithing. Now the brain is pithed with the same instrument used for the decerebration. The pithing rod is passed by way of the atlanto-occipital fissure through the foramen magnum into the cranium, a simple route which requires a minimum expenditure of time. If the brain alone is to be destroyed previous opening of skin or muscles is not necessary,—the instrument is introduced directly. If, however, the spinal cord is also to be pithed (all the thoracic section usually being destroyed), it is convenient to open the skin about 4 cm., transversely, below the occiput, and to cut the vertebral muscles till the spinal canal is reached. If this section of the muscles is limited to the region near the mid-line, with avoidance of the vertebral arteries, there is very little hemorrhage. To pith the spinal cord a heavy blunt wire is used, introduced by the same opening.

These operations usually required from 30 to 45 minutes. The preparation, with slight modifications, was used to test the different substances, previously mentioned, for their influence on the action of adrenalin and to investigate the effects of sympathetic stimulation. In the latter case both sciatics were frequently cut to rule out vasoconstriction of the legs.

In a few instances extirpation of the right adrenal and denervation of the left were performed aseptically, under ether anesthesia, and the animals were used four or five days later, when fully recovered.

We frequently omitted pithing the spinal cord, as we found a higher pressure than that mentioned by Elliott was desirable. This omission did not interfere with the sensitivity of the preparation but on the contrary sharpened the responses. According to our experience the optimum blood pressure is from 60 to 80 mm. Hg. The pithing of the brain may not be dispensed with, as otherwise the movements of the animal cause constant fluctuations in the blood pressure.

We did not find it necessary to cut the vagi, for their centers are destroyed by the pithing.

When we studied the responses of the denervated heart the animals were prepared as follows: In the morning (9 to 10 o'clock) the cat was lightly anesthetized with ether and both stellate ganglia and the upper thoracic sympathetic chains were removed through the right second intercostal

space, both vagi were cut in the neck, and a tracheal cannula inserted. Sometimes both adrenals were ligated by a lateral approach, in case denervation or removal of the adrenals had not been previously performed, as described above. Then the spinal cord was transected at the level of the 10th thoracic vertebra. In the afternoon the cat, well recovered, could be used without anesthesia or pithing; the arterial cannula was then inserted into the femoral artery.

A. *Means of "sensitizing" vascular responses to adrenalin.* The following agents were tested on the Elliott preparation:

1. Sodium bicarbonate (Snyder and Andrus, 1919; Snyder and Campbell, 1920; Collip, 1921; Hammett, 1922; Evans and Underhill, 1923; Lutz and Wyman, 1925), in varying doses of a 5 per cent solution given intravenously, merely influences the pressure responses to adrenalin insofar as it modifies the CO_2-carrying capacity or the pH of the blood. In hyperventilated animals sodium bicarbonate does not lead to any higher rise of blood pressure from adrenalin than before its administration. We therefore abandoned its use.

2. Glycocoll (Abderhalden and Gellhorn, 1923, 1924a, 1924b). Various doses of a 2 per cent solution given intravenously did not cause a greater response to adrenalin, when given either simultaneously or subsequently. On the contrary, large doses diminished the responses.

3. Calcium (Trendelenburg, 1916; Schmidt, 1921; Hülse, 1922). Different doses of a 5 per cent calcium chloride solution were tried. There was never a greater response to adrenalin after its administration. Large doses produced, on the contrary, a slight diminution in the effect.

4. Cocaine (Fröhlich and Loewi, 1910; Neubauer, 1913; Fischel, 1915; Santesson, 1919) invariably produced an increase in the pressure reaction to adrenalin. We therefore finally chose this substance as a sensitizing agent.

a. *Dose.* The larger the amount of cocaine injected, within limitations imposed by toxicity, the greater is the response to the same dose of adrenalin (see fig. 1). The doses used in our experiments varied from 3 to 10 mgm. per kilo. A 1 per cent solution of cocaine hydrochloride was injected intravenously, at the rate of 0.2 cc. per 5 seconds, until the heart slowed and the blood pressure fell.

b. *Action of cocaine on the heart rate.* When the maximal dose has been injected the rate falls considerably (from about 200 to 80 beats per minute). Then the rate rises slowly during about ten minutes and finally becomes constant at a higher level than before the cocaine (approximately 12 per cent faster).

c. *Action on blood pressure.* The blood pressure rises with small doses (2 mgm. per kilo). With larger amounts there is a sharp drop coinciding with the slowest heart rate. Finally, when the heart recovers, the blood pressure is slightly higher than before the injection.

d. *Toxicity of cocaine.* The susceptibility of animals is very variable; some cats are killed by 3 mgm. while others readily resist 10 mgm. per kilo. The rate of injection naturally has an important bearing on this resistance, slow injections being better tolerated. Adrenalin helps the animal to recover from the toxic action of cocaine. Animals with both adrenals ligated die with relatively small doses of cocaine (2 to 3 mgm. per kilo), while animals with intact adrenals or even with one extirpated and the other denervated will usually stand much larger doses.

e. *Absence of cocaine sensitization of the denervated heart.* The change in rate of the denervated heart following injections of adrenalin was found

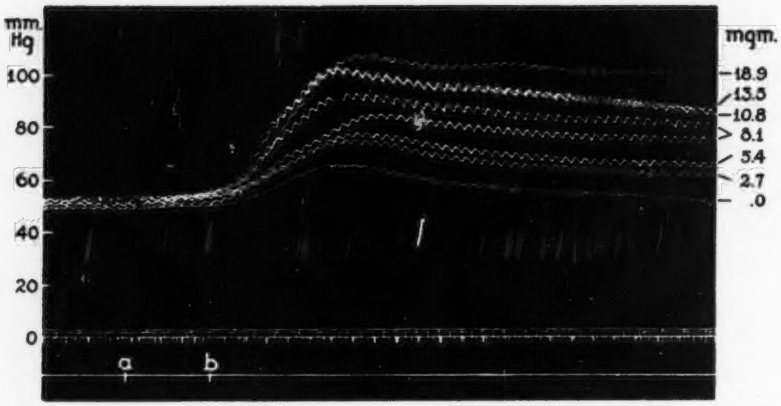

Fig. 1. Effects of the same dose of adrenalin (1 cc. of a 1:200,000 solution, injected between *a* and *b*) on the blood pressure in Elliott's preparation, after increasing doses (2.7, 5.4, 8.1, 10.8, 13.5 and 18.9 mgm.) of cocaine hydrochloride. Weight of the cat 2.7 kgm. The larger the amount of cocaine, the higher the rise in pressure. The lowest curve is that obtained before the cocaine injections. The preparation was kept slightly hyperventilated. The line of the time clock represents 0 pressure.

to be uninfluenced by cocaine. It will be shown later that cocaine does not influence the action of sympathin on the heart rate.

B. *Cocaine as a sensitizer for vascular responses to sympathin.* By the use of cocaine, definite rises in blood pressure appear when the sympathetic fibers innervating smooth muscle are stimulated.

Figure 2 shows the effects of sympathetic stimulation before and after injection of 20 mgm. cocaine into a 3.4 kgm. cat with the brain pithed and both sciatic nerves cut. Figure 3 shows a rise on stimulation of the sympathetic 40 minutes after injecting 30 mgm. cocaine hydrochloride into a 3 kgm. female. It will be noted that the blood pressure continues to rise after the stimulation ceases.

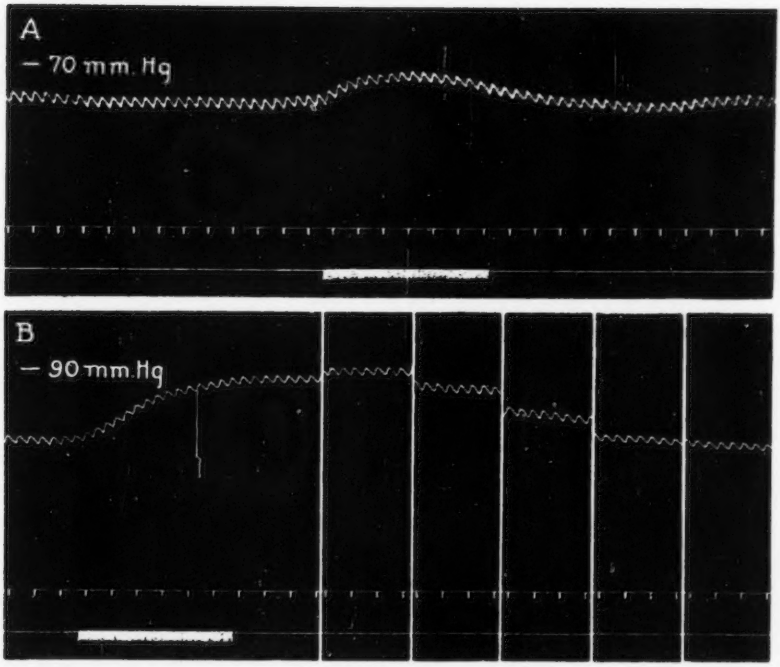

Fig. 2. Effects of stimulating for 30 seconds the lower end of the abdominal sympathetic chains, severed at the third lumbar ganglia, (A) before, and (B) after injecting intravenously 25 mgm. cocaine hydrochloride into a cat weighing 3.2 kgm. No anesthetic. Brain pithed, both sciatics cut. The first minute of record B is reproduced, and thereafter fifteen seconds of the record for each minute until, five minutes later, the original level is recovered. Lower line indicates 0 blood pressure.

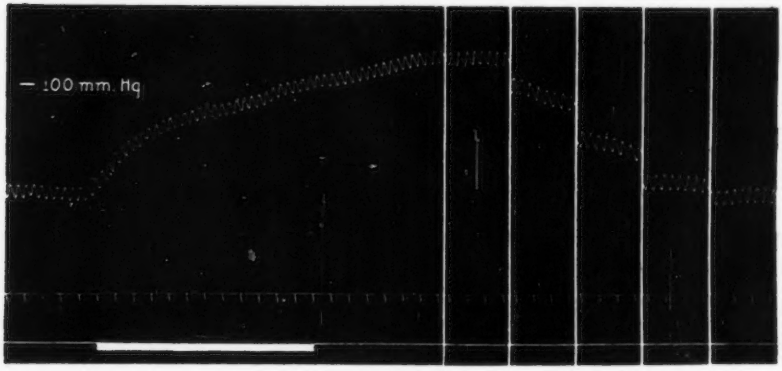

Fig. 3. Record showing continued increase of blood pressure after stimulating the lower end of the abdominal sympathetic, severed at the third lumbar ganglia, after injecting intravenously 22 mgm. cocaine into a cat weighing 3.4 kgm. Brain and dorsal spinal cord pithed. Left adrenal denervated and right removed. After the second minute of the record, the record for the first fifteen seconds of each subsequent minute is reproduced until the original level is recovered. The time line, registering five seconds, represents 0 blood pressure.

The sensitivity of animals differs considerably, the disturbing factors being low blood pressure and stimulation or injury of the sympathetic during the preparation. The first stimulus applied always elicits the highest response. Further stimuli occasion gradually diminishing reactions. Usually after three or four trials there is no further rise of blood pressure. The absolute rises varied from 20 to a maximum of 70 mm. Hg, when the pressure rose from 40 to 110 mm. Hg.

A tetanizing current from a "Harvard" inductorium, with a 10-volt primary current, was used. The strength of the stimulus was so gauged as to be barely perceptible on the tip of the tongue. The usual time of application was 30 seconds. The highest pressure generally appeared about 1 minufe after the stimulation ceased and there was a return to the original pressure about 5 minutes later.

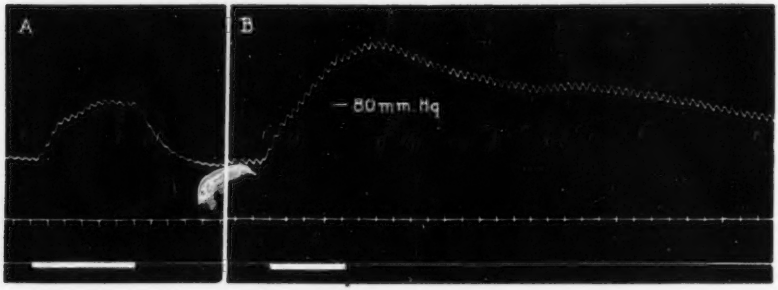

Fig. 4. Effects of occluding the circulation in the hind part of the cat by pulling a thread passed under the aorta and cava behind the renal vessels, (A) without and (B) with stimulation of the lower end of the cut abdominal sympathetic chains, after injecting 20 mgm. cocaine hydrochloride. Weight of cat, 2.8 kgm. Brain pithed, both sciatics cut. Lower line indicates 0 blood pressure.

Positive results were obtained only when the tail hairs were elevated.

C. *Effects of shutting off the blood supply from posterior end of animal with and without sympathetic stimulation.* These experiments were carried on in order to eliminate vasomotor reactions following sympathetic stimulation.

Merely occluding the circulation in the hind part of the animal produced a rise in pressure which lasted only during the occlusion; when the circulation was reëstablished the pressure fell to the original level within 10 seconds. Frequently the fall in pressure was below the base line but the return to the original level was prompt.

When, however, the sympathetic was stimulated while the circulation was occluded, the rise in pressure persisted for two or three minutes after the circulation was reëstablished and the descent was very slow. Sometimes there was a slight fall on relaxing tension on the vessels, followed by a

secondary rise immediately after such a fall. In these cases the pressure never fell below the original level. Figure 4 shows the results of these experiments.

D. *Quantitation of sympathin in terms of adrenalin.* It was found difficult to produce rises in pressure by adrenalin injections identical with those obtained on stimulation of the sympathetic, since the dose of adrenalin necessary to sustain a given rise is smaller than that requisite to produce it. Eight quantitations were made. The amount of adrenalin necessary to produce a rise in pressure equivalent to that obtained on stimulation of the sympathetic for 30 seconds was somewhat less than 1 cc. of adrenalin solution, 1/500,000, injected at the rate of 0.1 cc. in 5 seconds. The amount necessary to maintain this pressure was about 0.1 cc. (in 5 seconds) of a solution of 1/1,000,000. In order to match the curves more nearly

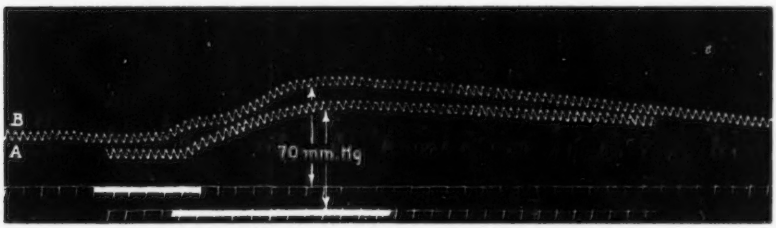

Fig. 5. Quantitation of sympathin in terms of adrenalin. (A) Rise in blood pressure on second stimulation of the lower end of the cut abdominal sympathetic chains, and (B) effects of the injection of 1.5 cc. adrenalin, 1:500,000. Cat weighing 3.1 kgm. Brain pithed.

it was therefore necessary to inject approximately 2.4 cc. of a 1/500,000 solution of adrenalin as follows:

0.6 cc. at a rate of 0.1 per 5 seconds	30 seconds
0.9 cc. at a rate of 0.1 per 10 seconds	90 seconds
0.9 cc. at a rate of 0.1 per 20 seconds	180 seconds
2.4 cc.	5 minutes

If the same amount is injected at a uniform rate of 0.1 per 10 seconds, the rise is greater and the fall sharper. The total amount of adrenalin thus injected is 0.000005 gram. This amount is somewhat greater than is actually represented by sympathin, because the rise of blood pressure due to sympathetic stimulation results in part, especially at first, from vasoconstriction of the tail region (see fig. 2A).

In figure 5 is presented an early attempt to match the records of the rise of blood pressure on the second stimulation of the lower end of the cut abdominal sympathetic chains, and the rise due to injection of adrenalin.

The amount injected in this instance was 1.5 cc. of a solution of 1:500,000. The records do not match perfectly because the adrenalin solution was injected at the uniform rate of 0.25 cc. per 5 seconds, and not gradually reduced in time as described above.

E. *Action on the heart rate.* We attempted to obtain changes in the rate of the acutely denervated heart with the technique described above, but cocaine did not lead to sensitization of this preparation and our results were negative. When the adrenal glands were tied off there was a very slight decrease (4 beats per minute in three cases) of the heart rate during stimulation, although the pressure rose. In one case when the adrenals were intact we had an increase of 8 beats per minute which appeared one minute after stimulation and lasted for two minutes. But these changes are not striking enough to permit definite conclusions.

DISCUSSION. The rise of blood pressure following the stimulation of the lower abdominal sympathetic could be due to the following reasons: 1, spread of the current with muscular contraction; 2, spread to the adrenal territory with secretion of adrenin; 3, vasoconstriction; 4, production of a substance having an adrenin-like action—sympathin. That this last is the actual case, is shown by the following considerations.

In a few instances there was slight spread of the stimulation to adjacent regions due to a deficient insulation of the electrodes or to fluid in the region where they were applied; muscular contractions ensued. The type of rise of blood pressure is then quite different from that appearing when only the sympathetic is stimulated. There is a sharp, high rise and a quick fall. These experiments were rejected.

There could not be a spread through the sympathetic chain, itself, toward the adrenals or the thorax, as the chains were always cut above the place of stimulation. Besides, the rises in pressure appeared even when the adrenals were extirpated.

Vasomotor phenomena were ruled out by the section of the sciatics and by the changes described when the circulation was occluded in the posterior end of the animal with and without sympathetic stimulation (see p. 370). Vasoconstriction quickly disappears on cessation of stimulation, whereas in our experiments there was a further rise and a very slow fall (see fig. 3). We interpret the first sharp rise in our curves as due to vasoconstriction but the remainder and main part of the curve cannot be due to these phenomena.

The curves obtained before cocaine or after several stimulations, very different from those in our successful experiments, belong to the type where only vasomotor effects come into play.

The fact that the rise in blood pressure following the first stimulation is always the greatest and that after 3 or 4 such stimulations no rise is obtained, is further evidence to support our interpretation that the rise is due

to sympathin, since vasoconstriction does not show such quick fatigue. Under these circumstances adrenalin will still produce its effects whereas additional injections of cocaine are ineffective in sensitizing the pressure responses to sympathetic sitmulations.

It is interesting that cocaine does not sensitize the heart to the action of sympathin. In our experiments it acted only as a sensitizer of smooth muscle.

SUMMARY AND CONCLUSIONS

The action of sodium bicarbonate, glygocoll, calcium chloride and cocaine on the rises in blood pressure produced by injections of adrenalin in Elliott's preparation was studied. Cocaine proved to be the only effective sensitizer among these substances.

Cocaine is more toxic to animals deprived of their adrenal glands than to animals with these glands present.

The higher the dose of cocaine, within limits set by toxicity, the more marked are its effects (see fig. 1).

Cocaine does not increase the effectiveness of adrenalin in accelerating the heart. It sensitizes smooth muscle to the action of adrenalin.

There is a close similarity between the action of cocaine as a sensitizer of smooth muscle to the influence of adrenalin and its rôle as sensitizer to the action of sympathin (see fig. 2). This evidence favors the view of the identity of adrenin and sympathin.

In animals with the brain pithed, the adrenals extirpated, the sciatics cut and the sympathetic chains severed at the 3rd lumbar ganglion, stimulation of the lower portion of the sympathetic, which causes contraction of pilomotor muscles, produces a rise in blood pressure after cocaine injection which continues after the stimulus has ceased and which disappears very slowly (5 minutes) (see fig. 3).

Shutting off the circulation in the posterior part of the animal by pulling on a thread passed beneath the cava and aorta behind the renal vessels causes a quick rise of pressure and a quick fall when the thread is released; stimulating the lower end of the abdominal sympathetic chains during the pull on the thread produces a persistance of the rise in blood pressure after the thread is released and the stimulus stopped (see fig. 4). The pressure falls slowly after this and is similar to the one mentioned above.

The quantitation of sympathin in terms of adrenalin shows (on the average) that in a typical instance it is necessary to inject 2.4 cc. of a solution of adrenalin of 1/500,000, the first 0.6 cc. at a rate of 0.1 per 5 seconds, the following 0.9 cc. at a rate of 0.1 per 10 seconds and the last 0.9 at the rate of 0.1 per 20 seconds, in order to match the rise in pressure occasioned by half-minute stimulation of the sympathetic. The total amount of adrenalin thus injected is 0.000005 gram (see fig. 5).

The store of sympathin is limited under the conditions of the experiment. The first stimulus is the most effective and the later stimuli (3 or 4) become rapidly incapable of producing any further results on the blood pressure.

We desire to take this opportunity to express our sincere appreciation to Prof. W. B. Cannon for suggesting this work and for his constant valuable criticism and encouragement during its development.

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ADRENALIN AND THE METABOLISM OF EXERCISE

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In exercise, the catabolism of the active muscles is greater than in rest so that adjustments of the circulatory and respiratory systems must enlarge the supply of oxygen for these combustion processes. The heart accelerates, the blood-flow to the viscera is curtailed, extra erythrocytes are released, and the ventilation of the lungs is made easier by dilatation of the bronchioles (Bainbridge, 1923). I mention these changes particularly because they are all adjustments which can be made by the sympathico-adrenal system (Cannon, 1929). No doubt adrenin is at least partially responsible for them since the work of Cannon and Britton (1926) has shown that adrenin is released even in very moderate activity.

Does the secretion of adrenin increase or decrease neuromuscular efficiency? Since adrenin is known to raise resting metabolism (McIver and Bright, 1924), the same result might be expected in exercise. In 1924 Boothby and Vidlicka brought forward evidence for this supposition. They showed that in man the total calorigenic effect of 0.5 mgm. of adrenalin in rest was 19 calories; at a slow walk it was 22 calories, at a moderate walk 17 calories and at a fast walk 60 calories. Scheuchzer (1928) reported that in rats adrenalin injections which raised the resting metabolism by 40 per cent increased that of exercise between 2 and 22 per cent. Rapport (1929) found that in dogs the extra heat produced by adrenalin in rest could not be used for muscular work.

My interest in this problem dates from the work of Campos, Cannon, Lundin and Walker (1929) in which it was shown that dogs "fatigued to a standstill" on a treadmill could be made to continue if they were given small doses of adrenalin. Was this due to some peculiar change in metabolism at this time?

Apparatus. In order to study this problem further, plans were made to measure the metabolism of exercise before and after adrenalin. Since rats had been made to run in revolving cages, it seemed quite possible that they might also run on a treadmill. An apparatus was therefore designed in which an animal was to be completely enclosed in a galvanized iron box with a moving belt at the bottom. Openings at the opposite ends allowed

the entrance and exit of air. As the rats had to be put in and taken out through the top, this could not be permanently sealed in place. Difficulties arose, therefore, in making sure that the top was tight after the animal was inside. At last the following procedure was adopted. The upper surfaces of the metal box were painted with a melted paraffin-vaseline mixture. When this had hardened and the rat was inside the box, the glass cover, heated in hot water, was put in place. This melted enough of the paraffin-vaseline mixture so that as soon as the top cooled, no leaks could possibly occur.

As the rats would not run voluntarily in the treadmill, a means of stimulating activity was devised. A hard-rubber plate with protruding metal points was placed at the back of the treadmill. When rats refused to run, they backed up against these points. If this stimulus was insufficient, then the points could be connected with an inductorium to give the animals a shock. Once a conditioned reflex had been established, the rats seldom needed stimulation.

To measure the metabolism of the animals, a closed-circuit type of apparatus was used. The carbon dioxide and water were absorbed by ascarite and dehydrite which were placed in Blount tubes (Lee and Brown, 1927). The carbon dioxide was determined by weight and the oxygen by volume. An electric blower forced the air through the absorbents and back to the animal chamber again, at a rate per minute equivalent to the volume of the system (1,000 cc.). The apparatus was checked for tightness before and after each experiment. Alcohol checks were difficult owing to the fact that a flame, equivalent to a rat's metabolism, is very small. On one day when a small flame was obtained, the following quotients were found: 0.663, 0.653, 0.655, 0.664, 0.653, 0.657, 0.653, 0.668, 0.668.

In considering the amount of work the animals should be required to do, different slopes and speeds of the treadmill were tried. In the end a 16 per cent grade with a speed of 2760 feet per hour was adopted. This rate corresponded to 10,000 revolutions of the recorder per hour. The results are given in the latter unit because the rate of the treadmill showed some fluctuations. The exercise was so moderate that it could be continued for long periods of time and yet it raised the metabolism to at least three times the basal level.

The adrenalin injections in exercising rats showed such interesting results that they were repeated with human subjects. In this case the treadmill ran at a rate of 100 revolutions or 64.3 meters per minute (about 2½ miles per hour). The metabolism was determined by the open-circuit method. Slopes from 5 to 23 per cent were tried. In most experiments the metabolism was raised to 6 or 8 times the basal level by this exercise.

RESULTS. *Rate of adrenalin absorption after subcutaneous injection.* Since it is difficult or impossible to inject rats intravenously, the sub-

cutaneous route was used. It was, therefore, important to know approximately how soon after such injections the maximum concentration of adrenalin would be found in the blood.

When large subcutaneous injections of adrenalin (0.02 mgm. per 100 grams) were given to rats, Cori (1929) has found by analysis that 65 per cent could be recovered after the first hour and 52 per cent after the second. The absorption was not complete after four hours. In attempting to check this work with smaller doses, I used a cat in which the heart, liver and adrenal glands had been denervated, and measured the speed of entrance of adrenalin into the blood by changes in the heart rate. This animal, even when it struggled, never showed a heart rate above 120 beats per minute. On the day of the experiment, salt solution was injected as a control. Thereafter at fifteen-minute intervals, the following heart rates were observed: 120, 116, 116, 108, 104, 104, 104, 104. The lower rates were probably due to decreasing activity of the animal.

TABLE 1

Averages of six experiments showing metabolic effect of small amounts of adrenalin (0.001 — 0.002 mgm. per 100 grams) in exercising rats

The metabolism is calculated per 10,000 revolutions of counter (per hour) and per 100 grams of rat.

	SALINE INJECTIONS		ADRENALIN INJECTIONS	
	Calories per 10,000 revolutions per 100 grams of rat	Respiratory quotients	Calories per 10,000 revolutions per 100 grams of rat	Respiratory quotients
Preliminary period	1.32	0.87	1.31 ±0.007	0.96
Period after saline or adrenalin	1.31	0.85	1.22 ±0.013	0.93
Succeeding control period	1.28	0.79	1.30 ±0.012	0.86

The cat then received a subcutaneous injection of adrenalin in a dilution of 1/100,000. The dose amounted to 0.02 mgm. per kilo. The subsequent heart rates were at fifteen minute intervals: 104, 128, 132, 136, 144, 148, 142, 146, 144, 144, 144, 138, 134, 134, 128, 120, 116, 114. This shows that the maximum concentration of adrenalin did not appear in the blood for at least one hour and a half and that some adrenalin was still present $3\frac{3}{4}$ hours after the injection.

Further proof of this slow absorption comes from experiments on human subjects in which the blood sugar rose slowly after the subcutaneous injection of adrenalin (see table 4).

These facts are emphasized because in the work to be discussed the effects of adrenalin were often delayed for one hour or more. The variations in the results probably depend upon whether the drug was injected near a blood vessel or not.

Metabolism of exercise after small doses of adrenalin. In order to determine the effects of adrenalin upon the metabolism of exercising rats, the animals had a preliminary run for at least two hours and during the latter part of this time their metabolism was measured. These determinations were continued until a steady state was reached. Then the animals were removed, given an injection of adrenalin and quickly returned to the treadmill. Metabolism estimations were again made after a lapse of fifteen minutes to allow the animal and apparatus to reach an equilibrium.

In many instances the effects of adrenalin were delayed for some time after the injection so that the results have been summarized in two tables. Table 1 contains the averages of the experiments in which the absorption was apparently rapid. It will be noted that following adrenalin, there is a fall in oxygen consumption with a later return to the previous level.

Table 2 contains the experiments in which the adrenalin effect was delayed for about two hours. At this time the metabolism was reduced.

TABLE 2

Average of six experiments showing delayed adrenalin effect (0.001 — 0.002 mgm. per 100 grams)

	CALORIES PER 10,000 REVOLUTIONS	RESPIRATORY QUOTIENTS
Preliminary period	1.36 ±0.013	0.88
Period after adrenalin	1.36 ±0.012	0.80
Period 2 hours after adrenalin	1.22 ±0.010	0.81

The results on rats were duplicated with human subjects. In each case the man was made to walk continuously for three or more hours. At the end of each half hour the expired air was collected in a spirometer and later analyzed for carbon dioxide and oxygen. After two or three preliminary periods, 0.5 or 0.25 mgm. of adrenalin was given to the subject and further metabolism measurements were made. The results of these experiments will be found in tables 3 and 5.

Of the nine experiments in which adrenalin was injected, two show no lowering of metabolism. In one of these, the determinations were probably not continued for a long enough time to get the result usually obtained; in the other, the adrenalin was given early in the exercise period before the subject was fatigued. As later discussion will show, fatigue evidently plays a part in this metabolic effect of adrenalin.

On the three starred days (table 3), blood sugars were determined (table 4) and it was found that a high blood sugar corresponded to a low metabolism on two of these days. On the other day, December 5, no lowering of metabolism was obtained.

In the periods when high blood sugars were found, the respiratory

TABLE 3

Metabolism in calories per 100 revolutions of treadmill (per minute) after each half hour of exercise (in man)

"A" indicates a subcutaneous injection of adrenalin (0.5 mgm.)

DATE	NOTES	1ST HALF HOUR	2ND HALF HOUR	3RD HALF HOUR	4TH HALF HOUR	5TH HALF HOUR	6TH HALF HOUR
January 22, 1931	G. C. R.* 5% grade	6.28	6.39	6.23	A 6.20	5.83	6.03
January 21, 1931	G. C. R. 5% grade	6.52	6.49	6.61	A 6.79	6.61	6.17
December 2, 1930	G. C. R. 5% grade	7.41 ?	6.93	6.90 A	6.46	6.53	
December 9, 1930	G. C. R.* 5% grade	6.67	?	A 6.77	6.28	6.46	
January 6, 1931	G. C. R. 12% grade	8.49	8.40	A 8.54	8.29	8.20	
December 17, 1930	C. W. H. 12% grade	7.22 ?	6.91	6.94 A	6.70	6.61	
December 5, 1930	G. C. R.* 5% grade	6.73	A 6.93	6.71	6.71	6.71	†
December 11, 1930	Control G. C. R. 5% grade	6.82	6.97	6.74	6.94	6.86	
December 19, 1930	G. C. R. 16% grade	8.92	9.28	9.14	A 9.23	9.27	†
January 13, 1931	J. O. P. 5% grade	5.81	5.23?	5.61	A 5.54	5.18	

* Blood sugars done on these days.

† No lowering of metabolism.

TABLE 4

Blood sugar after each half hour of exercise (in man)

"A" indicates a subcutaneous injection of adrenalin

DATE	1ST HALF HOUR	2ND HALF HOUR	3RD HALF HOUR	4TH HALF HOUR	5TH HALF HOUR	6TH HALF HOUR
January 22, 1931	100.5	112.3 ?	103.0	A { 103.0 / 105.2	113.0 / 112.3	104.7 / 105.2
December 9, 1930	101.0	85.1	A 98.5	113.0	99.5	
December 5, 1930	86.9	A 110.4	114.9	96.1	93.8	

quotients were below the previous range of variations (table 5). After adrenalin, in almost every case, the respiratory quotient showed a striking drop with a return toward the former level if the experiment was continued for a long enough time. This can only indicate a real increase in fat combustion since adrenalin given in exercise increases the lactic acid of the

TABLE 5

Respiratory quotients after each half hour of exercise (in man)

"A" indicates a subcutaneous injection of adrenalin

DATE	NOTES	1ST HALF HOUR	2ND HALF HOUR	3RD HALF HOUR	4TH HALF HOUR	5TH HALF HOUR	6TH HALF HOUR
January 22, 1931	G. C. R.* 5% grade	0.86	0.83	0.87	A 0.88	0.80	0.85
January 21, 1931	G. C. R. 5% grade	0.83	0.83	0.84	A 0.82	0.78	0.77
December 2, 1930	G. C. R. 5% grade	0.95	0.88	0.89 A	0.83	0.85	
December 9, 1930	G. C. R.* 5% grade	0.84	0.82	A 0.85	0.80	0.79	
January 6, 1931	G. C. R. 12% grade	0.88	0.79	A 0.80	0.80	0.78	
December 17, 1930	C. W. H. 12% grade	0.87	0.88	0.87 A	0.84	0.82	
December 5, 1930	G. C. R.* 5% grade	0.96	A 0.88	0.85	0.88	0.82	
December 11, 1930	Control G. C. R. 5% grade	0.80	0.81	0.81	0.82	0.83	
December 19, 1930	G. C. R. 16% grade	0.87	0.87	0.89	A 0.86	0.84	
January 13, 1931	J. O. P. 5% grade	0.87	0.87	0.86	A 0.87	0.82	

blood (Campos, Cannon, Lundin and Walker, 1929) and this means a blow-off of carbon dioxide and a high apparent respiratory quotient.

The pulse rates taken during these experiments showed no significant change.

The fall in metabolism following adrenalin as observed in my experiments

is not to be taken as contradicting the results of earlier work in this field. The experiments thus far cited show the effects of adrenalin after fatigue. If fatigue has not occurred, adrenalin exerts an entirely different effect upon metabolism. In a series of metabolism experiments done upon myself while exercising for ten minutes, it was found that results on control days would check within 2 per cent. If prior to this exercise adrenalin was given, my metabolism was increased by about 8 per cent. Table 6 gives these results. It will be noted that the metabolic increase averages 1.2 calories. In rest a similar injection raises the metabolism about 0.25 calorie per minute according to Sandiford (1920). The effect in rest is only one-fifth as great as that produced during exercise.

TABLE 6

Metabolism and blood sugar in exercise on a treadmill with a 23 per cent grade and rate of 120 revolutions per minute (in man)

DATE	NOTES	METABOLISM PER 120 REVOLUTIONS	RESPIRATORY QUOTIENT	BLOOD SUGAR	
				Just before	Just after
		calories			
October 15, 1930	Control	14.0	0.97	98	79
October 21, 1930	Control	14.2	0.93	97	82
October 24, 1930	Control	14.2	1.00	100	77
October 28, 1930	½ mgm. adrenalin	15.4	1.00	110	122
November 7, 1930	¼ mgm. adrenalin	15.4	0.96	119	99
November 17, 1930	½ mgm. adrenalin	15.0	0.95	126	112
November 28, 1930	Control	13.9	0.96	106	73
Average	Controls	14.1	0.97	100	78
	Adrenalin experiments	15.3	0.97	118	111

In these experiments where metabolism was increased the treadmill was set at a 23 per cent grade and the rate was 77 meters per minute (almost three miles per hour). This is considerably more work than was done in the long-continued exercise and for this reason perhaps should not be compared with it. Nevertheless, the work of Boothby and Vidlicka (1924) showed that adrenalin exerted a calorigenic effect even in moderate activity. Their subjects were not previously fatigued.

Metabolism of exercise after large doses of adrenalin. If large doses of adrenalin are given to fatigued rats, the calorigenic effect of the adrenalin appears. These results are given in table 7.

DISCUSSION. *Efficiency after adrenalin.* Since the work of La Franca in 1909, adrenalin injections have been known to increase the metabolism of resting animals. In fact, the adrenal glands are now considered to be

responsible for part of the resting heat as proved by the reduced metabolism of Addison's disease (Muirhead, 1921) and after inactivation of the adrenal glands in experimental animals (Aub, Forman and Bright, 1922; Barlow, 1924; Houssay and Artundo, 1929).

No satisfactory explanation of this calorigenic action is at hand in spite of the numerous studies which have been made. It appears to be a regular phenomenon—true for isolated organs and tissues and for the total organism as well. Evans and Ogawa (1914) found that the mammalian heart-lung preparation showed an increase of 65 per cent in oxygen consumption after adrenalin and Shaus and Bouckaert (1926) have noted a similar change in the frog's heart. Ahlgren (1925) found this to be true for skeletal muscle, and Abderhalden and Gellhorn (1926) substantiated it not only for whole but also for macerated muscle. The same results were obtained with heart, stomach and liver.

With the intact animal, using thermoelectric measurements, Caskey (1927) found a rise in the temperature of muscle after adrenalin and Caskey

TABLE 7

Averages of four experiments showing the metabolic effect of large amounts of adrenalin (0.025 mgm. per 100 grams) in exercising rats

	CALORIES PER 10,000 REVOLUTIONS PER 100 GRAMS OF RAT	RESPIRATORY QUOTIENTS
Preliminary period	1.34	0.81
Period after adrenalin	1.56	0.76
Final period	1.53	0.80

and Spencer (1925) confirmed Crile and Rowland (1922) who showed increased temperature in the brain under similar conditions. McIver and Bright (1924) have noted that partially eliminating the liver does not affect the calorigenic action of epinephrin, though Soskin (1927) states that in eviscerated or hepatized dogs, adrenalin does not prevent the usual decline in oxygen intake. Earlier work of Hunt and Bright (1926), however, shows a considerable effect of adrenalin after liver ablation. These workers believe that adrenalin has a general effect on tissue metabolism.

The calorigenic effect is not due to a release of carbohydrate, for Lusk (1928) points out that in a case of complete diabetes complicated with exophthalmic goiter, which raised the metabolism to plus 80 per cent, adrenalin given subcutaneously increased the basal metabolism to plus 149 per cent without any oxidation of sugar taking place. Also Boothby and Sandiford (1923) have found that an injection of sugar sufficient to cause a rise in blood sugar similar to that after adrenalin produced a very much smaller specific dynamic effect. Thus the evidence at hand would

lead to the conclusion that the calorigenic action of adrenalin in rest is not due to its action on selected tissues but is a generalized effect on the body as a whole and that this cannot be explained as caused by the carbohydrate plethora which adrenalin produces.

The experiments I have performed show that without previous fatigue, small doses of adrenalin produce a rise in metabolism and that after fatigue these same doses produce a fall in metabolism. Large doses of adrenalin always produce a rise in the metabolism of exercising rats. Evidently, then, with small doses fatigue throws out of action the calorigenic mechanism of rest.

Since so little is known of the calorigenic action of adrenalin, further discussion of this would be futile. The decreased oxygen consumption after adrenalin has two possible explanations. First, it may represent a real increase in efficiency. The increased blood-flow, the extra fuel and oxygen in the blood after adrenalin (Cannon, 1929) are all factors which might facilitate the functioning of fatigued muscles. It seems more probable that the decreased oxygen requirement does not represent a real increase in efficiency but a temporary means of lessening the work of the respiratory and circulatory systems; or to put it another way, the adrenalin may make possible a greater expenditure of energy in an emergency without a corresponding increase in oxygen intake. It is not hard to conceive of such an arrangement. The energy for muscular contraction is supplied by the conversion of glycogen to lactic acid. No oxygen is required in the process. If lactic acid could then be excreted, oxygen would not be used for any part of the exercise metabolism. To be sure, much energy would be wasted to the body but the situation would not differ in principle from emotional glycosuria. I hope soon to test this suggestion, that the kidneys may excrete more lactic acid after adrenalin is given to fatigued animals.

Note: The decreased respiratory metabolism of exercise which follows adrenalin injections lends support to the suggestion that the adrenal glands play a part in bringing about the changes of second wind.

Partition of foodstuffs after adrenalin. After adrenalin, there is a rise in blood sugar and blood fat (Himwich and Peterman, 1930). This indicates that the release from stores is more than keeping pace with the bodily requirements. With such a plethora of carbohydrates and fats, either foodstuff is available for the tissue combustions.

In studying the metabolism of isolated tissues, Wilenko (1912a) and Patterson and Starling (1913) found a greater utilization of glucose after adrenalin but this may be accounted for by the increased rate and force of the heart beat. Evans and Ogawa (1914) with a similar preparation noted a respiratory quotient of 0.775 during a control period and the same figure after adrenalin. They also found that when glucose had been added to the blood, adrenalin often caused a slight lowering of the respiratory quotient.

Determining the metabolism of the entire animal after adrenalin, La Franca (1909) found no change in the respiratory quotient and Wilenko (1912b) obtained similar results even when adrenalin was given after an intravenous injection of glucose. He believed that adrenalin lessened the ability of the body to burn sugar. Lusk and Riche (1914) found contrary evidence in similar experiments on dogs. Hári (1912) noted a fall in oxygen consumption but a rise in the respiratory quotient. Fuchs and Roth (1912) concluded that adrenalin increased the carbohydrate combustion.

One of the most significant papers in this field is that of Krantz and Means (1927) who determined the respiratory quotients of normal and obese patients. They found an immediate rise in the respiratory quotient due no doubt to the fact that lactic acid was poured into the blood by adrenalin (Cori and Cori, 1928) and caused a blowing off of carbon dioxide. The later respiratory quotients in the normal patients show a much higher carbohydrate combustion than in the obese. The authors explain this result by saying that obese individuals have less stored glycogen and more fat available and that the metabolic stimulant causes the oxidation of whatever food is at hand.

The Coris' work (1928) has also shown no increased carbohydrate combustion after adrenalin.

The results which are reported in this paper are consistent with the inference that if adrenalin is given late in a fatiguing exercise, the body usually goes onto a larger fat metabolism.

SUMMARY

A treadmill for rats has been devised and the metabolism of rats during activity has been determined (see tables 1 and 2).

Small doses of adrenalin (0.001–0.002 mgm. per 100 grams) given to fatigued rats have been found to lower their respiratory metabolism in exercise (see tables 1 and 2).

In man, as in the rat, physiological amounts of adrenalin (0.5 mgm. per person) given after fatigue cause a lowering of the respiratory metabolism of exercise (see table 3).

Without previous fatigue, the metabolism of exercise is increased by a similar amount of adrenalin (see table 6).

The respiratory quotients of exercise were found to be lowered by adrenalin (see table 5).

I wish to express my thanks to Dr. W. B. Cannon for suggesting this problem and offering advice as the work was carried along. Miss E. N. Ingalls has made all the blood sugar determinations.

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THE EFFECT OF CALCIUM CITRATE AND CARBONATE UPON THE EVACUATION OF A PROTEIN FROM THE STOMACH OF THE ALBINO RAT AND THE pH OF THE CONTENTS[1]

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Data have been accumulated on the evacuation time of various foodstuffs from the stomach and the effect of these on the acidity during periods of gastric digestion. These investigations have been carried out on the human, the dog and the cat, but little attention has been given to the albino rat except in the case of hydrogen ion observations as in the work of Abrahamson and Miller (1925) and Redman, Willimott and Wokes (1927). Little or no attention has been paid to the effect of the feeding of calcium compounds on the pH of the stomach contents and on the extent of evacuation in any species. In the work which follows, the albino rat, so largely used in feeding trials, was used as the experimental animal.

This experiment was designed to determine the effect of calcium citrate and carbonate, when fed with a protein, on the extent of the evacuation of the stomach contents and on the pH of the contents at the end of a given period of time. Coagulated egg white was selected as the protein because of its uniform character, which allowed reproducibility of the experimental diet from day to day.

EXPERIMENTAL. Finely minced, coagulated egg white was fed with and without calcum citrate and carbonate incorporated at different levels. The two forms of calcium were introduced into the diet in quantities sufficient to supply equivalent amounts of calcium from each source at two levels. The amounts used are shown in table 1.

Adult white rats, 10 to 12 months of age, from the breeding colony, were placed in cages equipped with false bottoms to prevent consumption of

[1] Prepared from a dissertation submitted by F. G. McDonald, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy, Rutgers University, June, 1929.

Journal Series paper of the New Jersey Agricultural Experiment Station, Department of Agricultural Biochemistry.

[2] Calcium Citrate Industrial Fellow. Now at Mead Johnson and Company, Evansville, Indiana. This investigation was conducted under a fellowship grant from Charles Pfizer and Company, Inc., Brooklyn, N. Y.

feces, and starved for 24 hours. Distilled water was available but was removed when the food was introduced. At the end of this period a known amount of the food under investigation was placed before the animal for 15 minutes, after which it was removed and weighed approximately to the second decimal place. If the animal ate sufficiently of the food, it was allowed to remain in the cage for 2 hours, then anesthetized and the abdomen opened immediately. The stomach was ligatured just above the pyloric valve and at the cardiac orifice, and quickly dissected out. It was then quickly washed with distilled water, and the whole of the contents removed and weighed.

In general it was not possible to obtain a sufficiently large sample of the liquid phase for the hydrogen ion determination unless water was added. According to Kahn and Stokes (1926) dilution of samples of human gastric juice (1:10), when the pH was below 3.5, caused a diminution of the hydrogen ion concentration. Trials in this laboratory showed that the stomach contents of the white rat are not well buffered and therefore care

TABLE 1

Weights of calcium compounds per 100 grams of food and calcium equivalents

SALT	WEIGHT OF SALT	CALCIUM EQUIVALENT
	grams	*grams*
Calcium citrate	10.00	2.107
Calcium carbonate	5.26	2.107
Calcium citrate	1.92	0.404
Calcium carbonate	1.00	0.401

was taken to add a minimum amount of distilled water. Since the amount of liquid phase present in a sample of stomach contents was not known, and could not be determined before the hydrogen ion concentration was measured, it was necessary to apply the technique of adding just enough freshly distilled water, a few drops, to make a stiff paste. The pasty material was centrifuged for a few seconds and the few drops of supernatant liquid triturated with quinhydrone. The whole of the above procedure was carried out with the greatest possible speed in order to avoid undue exposure of the contents and the possible loss of CO_2. As pointed out by Redman, Willimott and Wokes (1927), protein and salt errors "may be responsible for differences of 0.2 to 0.5 pH" but in the present investigation there was no way of eliminating these as possible sources of error In order to avoid the influence of an unconscious change of technique on the part of the experimenter all of the individuals on a given diet were not used in sequence, but small groups were used successively on each diet until the total number of animals had been studied.

The quinhydrone electrode was used in the determination of the hydro-

gen ion concentration. The type of electrode vessel employed was designed after the straight tube capillary vessel type C, described by Cullen and Biilmann (1925), and had as an electrode a 24 carat gold wire, as recommended by Cullen and Earle (1928). This type of electrode permits the working with small volumes of liquid and minimizes the escape of gases. The modification made in the electrode consisted of soldering a piece of gold wire, no. 28, 3 inches long to no. 18 copper wire. The wire was placed in a glass tube of small diameter drawn to a fine point with about 1.5 inches of the gold wire projecting out through the drawn end. The wire was cemented into the tube by means of soft DeKhotinsky cement, with the upper part of the projecting portion covered with the cement. The upper part was covered so that the portion of the wire involved in making the measurement would not be subjected to a change in concentration of CO_2 due to escape of this gas at the surface (Cullen and Biilmann, 1925). A piece of rubber tubing was fitted near the end of the electrode so that it served the purpose of a plunger and a solution could be drawn into the capillary of the electrode vessel. Agreement closer than 2 millivolts between successive determinations was not always obtained so that at least 2, and if possible 4 or 5 determinations were made. The readings were made at about 25°, using a Leeds and Northrup type K potentiometer, with lamp and scale galvanometer.

RESULTS AND DISCUSSION. The percentage of evacuation was calculated from the food intake and the weight of the material recovered from the stomach at the end of a 2-hour period. The values are shown in chart 1. The moisture content of the material fed was approximately the same before it had entered and after it had remained in the stomach for 2 hours. Thus, for coagulated egg white the moisture content was 87.7 per cent $\pm$ 1 per cent for an average of 8 determinations, and it was 87.4 per cent $\pm$ 1 per cent for an average of 8 determinations, after the egg had remained in the stomach for 2 hours. Moreover, the same agreement was found between the moisture contents when the calcium salts were incorporated with the egg material.

Probable error calculations in the case of the two average percentage evacuations, chart 1, between which there is the greatest difference, namely, groups 1 and 2, show that the chances are only 21 to 1 that the difference is significant. Hence the data only suggest that a real difference exists in the extent of evacuation of the coagulated egg white with and without calcium citrate and carbonate at the two levels used. Although the differences are not marked, it is of interest that there was a delay in evacuation when the calcium compounds were fed. In this connection it should be noted that 1.92 per cent calcium citrate or 1 per cent of calcium carbonate reduces the percentage evacuation in 2 hours to about the same degree as does 10 per cent of the former or 5.2 per cent of the latter.

Although the pH of the stomach contents was higher when calcium carbonate was supplied than when calcium citrate was used, as will be noted later, yet the extent of disappearance from the stomach is approximately the same, indicating that no relation exists in this instance between the pH of the contents and the evacuation time. This observation is in agreement with the present day concept of the emptying mechanism of the stomach (Murlin, 1930).

The distribution of pH values in chart 2 indicates definitely that 10 per cent calcium citrate and 5.2 per cent calcium carbonate have caused a marked change in hydrogen ion concentration of the stomach contents

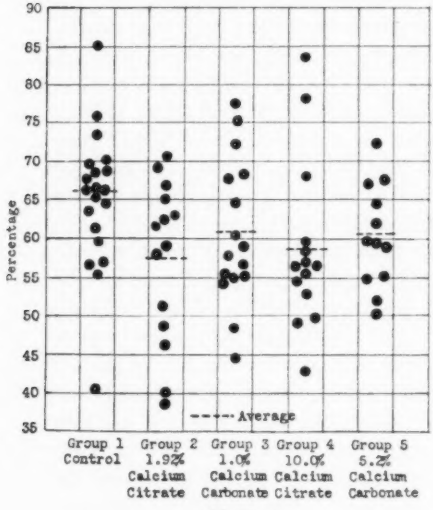

Chart 1

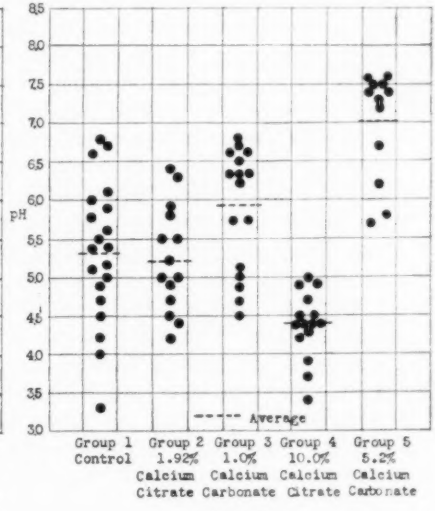

Chart 2

Chart 1. Percentage of coagulated egg white evacuated from the stomach of the albino rat in a two-hour period.

Chart 2. Effect of calcium carbonate and calcium citrate on the pH of the stomach contents of the albino rat.

from that observed in the case of the control animals, group 1. The average pH when 10 per cent calcium citrate was used was 4.4 as compared with 7.0 when 5.2 per cent calcium carbonate was incorporated. The difference between the group which received 1.0 per cent $CaCO_3$, group 3, and the control group is not as marked as that in the case just cited. Probable error calculations show that the chances are 21 to 1 that the difference between groups 1 and 3 may be significant. Hence the lower level of calcium carbonate had only a slight effect and the lower level of calcium citrate no effect on the pH of the stomach contents. It is also

to be noted that the pH of the contents from individuals varies within each series, chart 2. This is to be expected as the response of the stomach of the individuals no doubt varies somewhat.

Redman, Willimott, and Wokes (1927) report the pH of the stomach as 3.0 to 5.4, average 4.2, using an electrometric method, for a series of 9 adult rats when receiving different normal diets. Abrahamson and Miller (1925) fed adult rats a diet of lean beef only and obtained values for the stomach of 2.6 to 4.0 with an average pH of 3.3 for a series of 12 animals. They employed the colorimetric instead of the electrometric method. The values of the pH of the stomach contents of rats reported by the above workers are somewhat lower than those reported herein, obtained as the average of a series of 12 animals receiving coagulated egg white. This might very well be expected because boiled egg seems to induce a smaller secretion of gastric juice with a resulting lowering of the acidity, than does beef, as pointed out by Hawk, Rehfuss and Bergeim (1926).

In the case where 10 per cent calcium citrate was fed a pH value higher than 5.3, obtained when no calcium salt was incorporated, might be expected rather than a lower one, because in the reaction of calcium citrate with hydrochloric acid, the acid produced, citric acid ($Ka = 8.2 \times 10^{-4}$, 25°, first dissociation) (Walden, 1892), is much less dissociated than hydrochloric acid of the same concentration. It is possible that calcium citrate or citric acid has a stimulating effect on the production of hydrochloric acid, although Foster and Lambert (1908), working with dogs, report that citric acid does not have the property of increasing the flow of gastric juice. Such an effect could not be attributed to the calcium chloride produced because this compound would also be formed when calcium carbonate was used. The slight dissociation of the carbonic acid and the tendency of carbon dioxide to escape would, of course, explain the increase in pH when calcium carbonate is employed. The hydrogen ion concentration of the contents would not be expected to be as low in the case of the calcium citrate feeding as in that of the calcium carbonate because the organic acid produced is not volatile and is more highly dissociated than carbonic acid. Northrop (1919) has shown that at equal hydrogen ion concentration the rate of pepsin digestion of egg albumin is the same in solutions of hydrochloric and citric acid, in vitro.

SUMMARY

1. The extent of the evacuation of coagulated egg white from the stomach of the white rat was greater in the absence of the calcium compounds, calcium citrate and calcium carbonate, and the data suggest that the difference is a significant one. Both compounds reduced the amount of the evacuation when fed either at a high or low level.

2. When the coagulated egg white was fed without the addition of a

calcium compound the pH of the contents was 5.3. Upon incorporating 10 and 1.92 per cent calcium citrate in the food it was found that the higher level lowered the pH to 4.4 whereas the lower level had no effect. Calcium carbonate introduced into the test meal instead of the citrate, in quantities equivalent in calcium content to that of the citrate increased the pH of the contents to 7.0 in the case of the higher level, whereas the smaller dosage of calcium carbonate raised the value only 0.6 pH over that of the control.

3. The evacuation of the material from the stomach took place to approximately the same extent when a pH of 7.0 was obtained as when a value of 4.4 was observed.

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ATTEMPTED AUTOTRANSPLANTATION OF THE ADRENAL CORTEX

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Transplantation of tissues has been attempted with varying degrees of success for a long time, in a variety of species. Clinically, skin and bone have been successfully transplanted and experimentally a number of tissues, such as thyroid and parathyroid (Manley and Marine, 1916), stomach (Ivy and Farrell, 1925), and pancreas (Ivy and Farrell, 1926) have been autotransplanted. Homotransplants are more rare, although such grafts have been reported for the parathyroids (Erdheim quoted by Dragstedt, 1927) and hypophysis (Allen, 1921). It is doubtful whether functioning heterotransplants have ever been made.

Attempts to transplant the adrenal cortex have been made repeatedly, but on the whole the few "takes" reported are open to criticism. Stilling (1905) reported success on histological evidence alone, as did Busch et al. (1905) (1908), who in addition did cite two instances where the physiological criteria of success were fulfilled On a statistical basis Sundberg (1925) reported "takes" in rabbits, about twenty per cent of which, however, have accessory cortical tissue adequate to support life. True transplants by the pedicle method, so successful in both the human skin and also the gastric mucosa (Ivy and Farrell, 1925) and pancreas (Ivy and Farrell, 1926), have failed in the hands of Haberer and Stoerk (1918) and Oldberg (1929). Zwemer (1927) and others have also been unable to produce growing transplants.

METHOD. The dog was chosen as the experimental animal because accessory adrenal glands are very rare (Stewart and Rogoff, 1928). This criterion of success was adopted: the transplant must maintain the animal alive and well after removal of both glands; the animals must die with the characteristic symptoms when the graft is removed; there must be histological evidence of cortical tissue in the transplants; there must be no accessory cortical tissue found on careful search at autopsy.

Transplants were attempted to the thyroid, because of its vascularity, and the omentum, testis and kidney, because of previous suggestions of success in these tissues. In one series emulsions of cortical tissue were injected into the splenic vein, the aim being to make multiple small trans-

plants to the liver, such as occurs presumably in carcinoma metastases. Furthermore, Manley and Marine (1916) have made autografts of the thyroid into blood vessels, using the jugular vein.

At operation under morphine-ether anesthesia the host tissue was exposed before doing the adrenalectomy. For this latter a three to four inch incision was made in the abdominal wall about an inch lateral to the border of the rectus abdominis muscle and the gland approached from its ventral surface. The right gland was dissected away with an orangewood stick, care being exercised not to injure the splanchnic nerve. When the gland was freed except for the craniad portion, where the artery enters, the lumbo-adrenal vein was ligated proximal and distal to the gland, and the excision completed as rapidly as possible. The adrenal artery rarely required ligation. Tamponade for a minute or two was usually sufficient whenever the artery bled.

While one operator prepared thin slices of cortex from the excised gland the other incised the host organ. Sometimes before implantation the pieces of cortex were placed in warm Ringer's solution through which oxygen was bubbled for two or three minutes. The slices were inserted in place, usually within about five minutes from the time of occlusion of the gland's vessels, and the host tissue sutured. The sutures never passed through the transplant. Lastly, the abdominal wound was closed.

After experimenting with recovery periods varying from one to eight weeks, four weeks was selected as the optimum. At the second operation the left gland was excised as rapidly and with as little trauma as possible, using essentially the above described technique. The time elapsing between making the incision and finishing suturing was about twenty minutes. In some instances the transplantation was done at the second operation, and sometimes at both operations.

The animals, which had previously been on a diet of meat, bread, bone meal, and water, were now given milk, sugar, bread, bone meal and water, and were kept in a warm room until death. Autopsy was always done and the site of the transplant examined carefully. In approximately the first half of the dogs run, death often occurred within three days. Surgical trauma was undoubtedly a factor in these early deaths, which presumably would have occurred even if there was present a growing transplant producing the minimum physiological action necessary for life. Histological studies of the site of the transplantation were therefore made in these. If the microscopic evidence were positive, little could be concluded. But if it were negative, it would be safe to assume that death would have occurred in a few days even had there been no surgical trauma. Later in the work, when animals lived four to six days and death could be attributed to adrenal insufficiency, histological studies were not made.

Nodules suspected of being accessory cortical tissue were also examined

microscopically, but no rests were found in over 150 dogs of this and other series. The viscera of the chest and abdomen were examined routinely, grossly and sometimes microscopically. Throughout the experiment control animals were operated and treated as described above, except that no transplantation was done.

RESULTS. In most cases complete absorption of the transplant with scar tissue formation was seen. In a few instances there were microscopic suggestions of cortical tissue, but vascularization was never observed. These few anatomical "takes" were entirely inadequate to support life, either because they were too small or else because they were not physiologically active. By physiological criteria the transplants never took. Table 1 gives a summary of the results. It will be seen that the average survival time for animals with transplants was slightly under that of the controls.

TABLE 1

Average time of survival of transplanted and control dogs following second adrenalectomy

SITE OF TRANSPLANT	ALL DOGS OF SERIES		ONLY DOGS SURVIVING AT LEAST 3 DAYS		LONGEST SURVIVAL TIME
	Number of dogs	Days survival	Number of dogs	Days survival	
					days
Thyroid	19	3	8	4.5	6
Thyroid and omentum	3	2.5	1	3	3
Kidney	11	2.5	3	5	10
Emulsions to liver	5	5	5	5	7
Testis	8	4.5	6	5.5	7
All transplants	46	3.4	23	5	10
Controls	14	4	6	5	8

The third and fourth columns of the table give the results only on those dogs which survived at least three days post-operatively. These dogs may be considered to have died from adrenal insufficiency, and not from surgical trauma. However, the entire series of sixty dogs is of value, although 31 of them died in one to three days, for in these the histological evidence for failure is adequate. Because averages may sometimes be deceptive there is also given the longest survival time in each series.

Incidentally, we confirm the findings of Stewart and Rogoff (1926) and others regarding the behavior of dogs after double adrenalectomy: post-operative depression, recovery from this with resumption of eating and other normal activities, and then a rather sudden decline with refusal of food, vomiting of bile tinged fluid, bloody diarrhea, asthenia, "hallucinations," and death from respiratory failure. Pathological findings were also essentially those of previous workers: hemorrhagic infiltration of the gas-

tro-intestinal mucosa and pancreas, blood in the intestinal lumen, and gastric ulcers.

CONCLUSIONS

Using the methods described we were uniformly unsuccessful in attempts to autotransplant the adrenal cortex of 46 dogs to the thyroid, omentum, testis, kidney and liver.

We appreciate greatly the helpful suggestions and advice given us by Dr. A. J. Carlson.

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